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Clinical and Experimental

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DEMONSTRATION OF A THROMBOCYTOPENIC FACTOR IN THE BLOOD OF PATIENTS WITH THROMBOCYTOPENIC PURPURA

WILLIAM J. HARRINGTON, M.D., VIRGINIA MINNICH, M.S.,
JAMES W. HOLLINGSWORTH, M.D., AND CARL V. MOORE, M.D.
ST. LOUIS, MO.

THE presence of a thrombocytopenic factor has been demonstrated in the blood of eight out of ten patients with idiopathic thrombocytopenic purpura. Transfusion of 250 c.c. of plasma or 500 c.c. of whole blood from these eight patients into nonthrombocytopenic recipients caused a prompt, dramatic fall in the platelet level. Two of the recipients developed the hemorrhagic manifestations characteristic of purpura hemorrhagica. In two of three patients with secondary thrombocytopenic purpura the platelet reducing factor could not be demonstrated.

Current concepts of the pathogenesis of idiopathic thrombocytopenic purpura postulate that the low platelet count results either from a suppression of megakaryocyte activity,[1, 2] or from an increase in platelet lysis[3] or phagocytosis.[4] Recent studies have also indicated that platelet agglutinins may be found in the blood of patients suffering from thrombocytopenia of the idiopathic variety[5] or caused by sedormid sensitivity.[6] These latter observations and those contained in the present report: (a) show that a circulating thrombocytopenic factor is of importance in the mechanism responsible for platelet reduction in many cases of purpura hemorrhagica, and (b) suggest similarities in the pathogenesis of idiopathic thrombocytopenic purpura and of acquired hemolytic anemia.

MATERIAL AND METHODS

Blood from thirteen patients with thrombocytopenic purpura and from seven nonthrombocytopenic subjects was collected into acid citrate dextrose solution. In all but a few instances, a volume of 500 c.c. of freshly collected whole blood or its plasma equivalent was transfused into nonthrombocytopenic recipients who were either healthy volunteer subjects from the laboratory or patients with inoperable malignant neoplasms. The number of circulating platelets,[7] red cells, and white cells was counted frequently during the first three or four hours after transfusion, and daily thereafter until values returned to control levels. In some of the recipients, repeated observations were made of: bone marrow morphology, bleeding time,[8] capillary fragility,[9] silicone clotting time,[10] clot retraction, and prothrombin consumption.[11] Serum for measurement of prothrombin consumption was obtained from blood placed in silicone tubes and kept for twenty-four hours at 37° C. With this technique, 35 to 45 per cent of the prothrombin is consumed in the blood of normal subjects.

RESULTS

The age and sex of each patient, the diagnosis, the acuteness or chronicity of the disease, and the response to splenectomy are summarized in Table I.

From the Department of Internal Medicine, Washington University School of Medicine.
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TABLE I.

PATIENT	EFFECT OF SPLENECTOMY	THROMBOCYTOPENIC FACTOR	
		BEFORE SPLENECTOMY	AFTER SPLENECTOMY
Idiopathic Thrombocytopenic Purpura			
Acute			
1. R. C., 9 yr., M	Remission	+	–
2. E. T., 49 yr., F	Remission	+	+ (5)*
3. F. C., 45 yr., F	No platelet rise in 8 mo.	–	+ (60;200)*
4. S. H., 19 yr., F	Slow platelet rise after 2 wk. Relapse 2 wk. later	0	–
Chronic			
5. H. S., 42 yr., M	No platelet rise in 4 mo.	+	+ (120)*
6. L. A., 36 yr., F	No platelet rise in 2 mo.	+	–
7. J. B., 61 yr., M	No platelet rise in 6 wk.	0	–
Recurrence after Splenectomy			
8. M. H., 33 yr. F	Improved but continued thrombocytopenia 12 yr.	–	+ (12 yr.)
9. L. P., 63 yr., F	Relapse in 3 mo.	+	+ (6;18)*
10. G. M., 30 yr., F	Relapse in 4 yr.; remission with cortisone	+ (before cortisone)	0 (after cortisone)
Secondary Thrombocytopenic Purpura			
Chronic lymphocytic leucemia			
11. W. D., 60 yr., M	Splenectomy after subarachnoid hemorrhage. Died in 5 days—no platelet rise.	+	–
12. D. B., 55 yr., M	Subarachnoid hemorrhage 2 wk. post splenectomy No platelet rise. Died	0	–
Hypoplastic Anemia			
13. N. B., 6 yr., F	Slight improvement Minimal platelet rise in 6 wk.	0	–

+, present; 0, not demonstrated; –, not tested for.

*Figures in parentheses indicate days after splenectomy on which blood was drawn for the test.

Blood or plasma from nine of the thrombocytopenic subjects regularly produced a prompt, and in some instances a profound decrease in the number of circulating platelets in the recipients. Blood was usually obtained from each patient on several different days so that the effect of these transfusions was observed in thirty-five separate studies. The decrease in platelets was often apparent within thirty to sixty minutes, became maximal within two to three hours, and persisted for four to seven days. The results of one transfusion from each of the eight patients with idiopathic thrombocytopenic purpura who showed this effect are charted in Fig. 1. Blood from W. D., a man with chronic lymphocytic leucemia, produced similar changes. Nonhemolytic transfusion reactions frequently occurred after administration of the blood or plasma; these varied from mild malaise to frank chills with fever, backache, and, in one recipient, convulsions. It is interesting that in several instances transfusions of platelet-rich plasma into the donor patients with thrombocytopenia also caused similar reactions.

The four patients whose plasma failed to cause thrombocytopenia when transfused were: two of the individuals with idiopathic thrombocytopenic pur-

pura (Nos. 4 and 7, Table I), one patient with chronic lymphocytic leucemia (No. 12), and the child with hypoplastic anemia (No. 13). These negative results were verified by second observations in two of the cases. Control observations were made by transfusing blood or plasma from seven nonthrombocytopenic donors (Fig. 2). In only one instance did a significant decrease occur in the

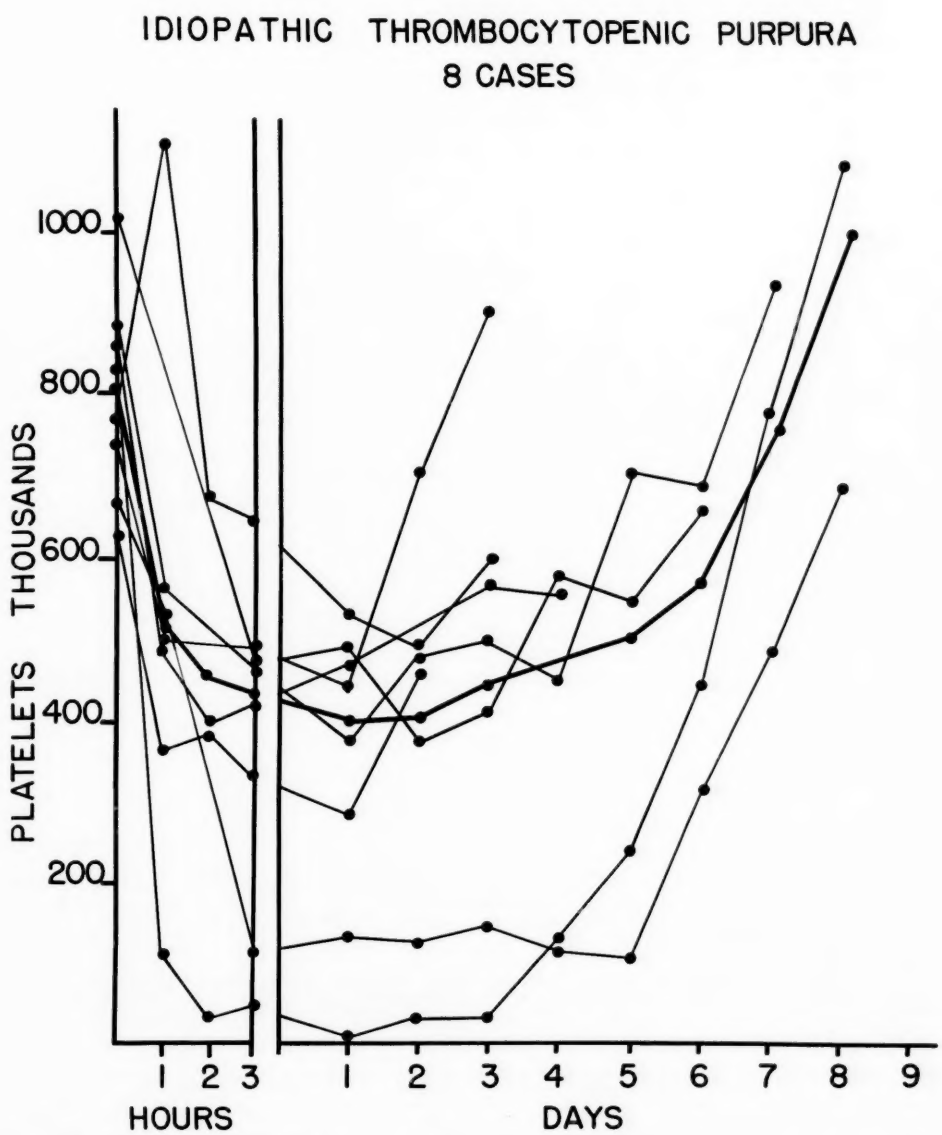

Fig. 1.—Thrombocytopenic effect produced by transfusing 500 c.c. of citrated whole blood or its plasma equivalent from eight patients with thrombocytopenic purpura. Transfusions were given at "0" time. Recipients were healthy laboratory workers or patients with inoperable carcinoma. The mean effect is represented by the heavy line.

platelet count of a recipient; this subject was a young physician who had developed signs of a severe upper respiratory infection within twenty-four hours after he had been transfused.

If the life span of platelets is four to seven days,[12, 13] then the thrombocytopenia induced in this study was apparently due to rapid disappearance of plate-

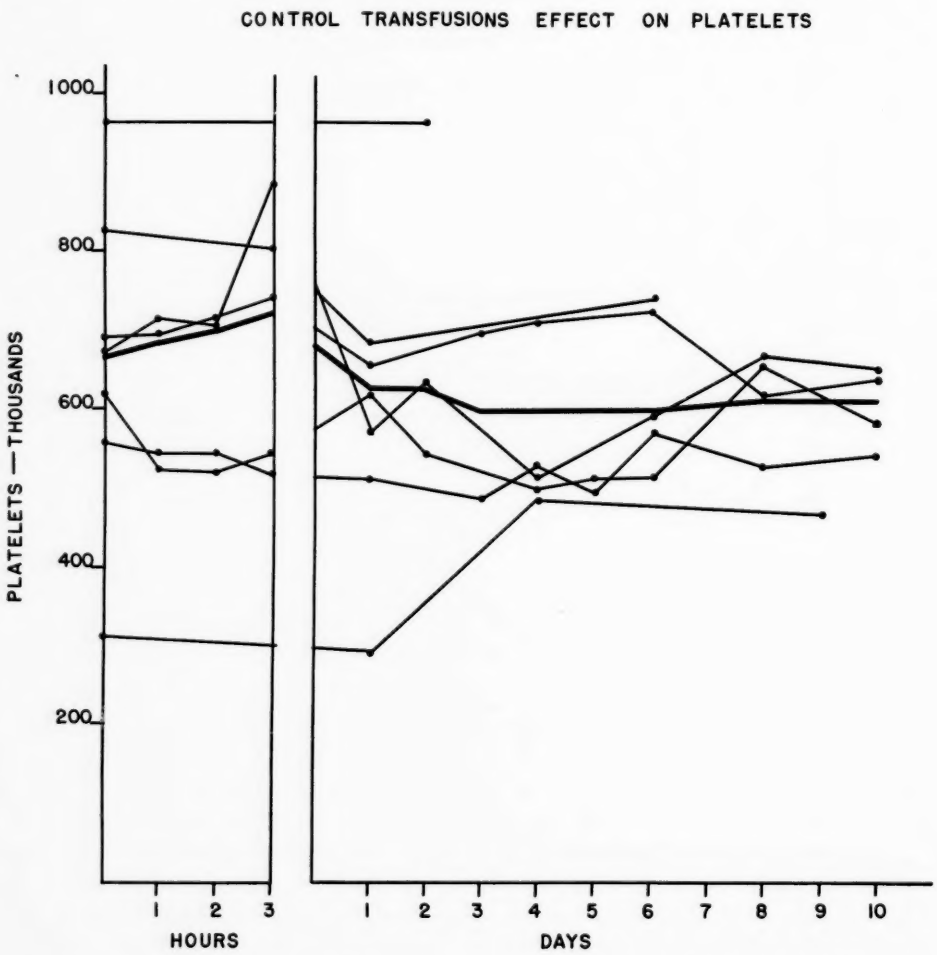

Fig. 2.—The failure of transfusions of 500 c.c. of whole blood from control subjects to produce a significant fall in the platelet count of recipients. The greatest fluctuation observed was in a normal subject who developed an upper respiratory infection during the period of study.

lets from the circulating blood and not merely to inhibition of platelet formation. Furthermore, the entire clinical and laboratory syndrome of thrombocytopenic purpura was induced in two recipients within the first few hours. Both of these subjects bled from the gastrointestinal tract in addition to showing petechiae and purpura. These manifestations persisted for four to seven days, paralleling the duration of the low platelet count. Fig. 3 shows the evolution

of the abnormal laboratory data in a recipient in whom thrombocytopenia was induced twice by transfusion. When the platelet reduction was greatest, bleeding time was found to be prolonged, and the prothrombin consumption was abnormally low. Why capillary fragility was increased after the first transfusion but not after the second is not clear. Bone marrow megakaryocytes showed the characteristics of immaturity during the thrombocytopenic period. Initial

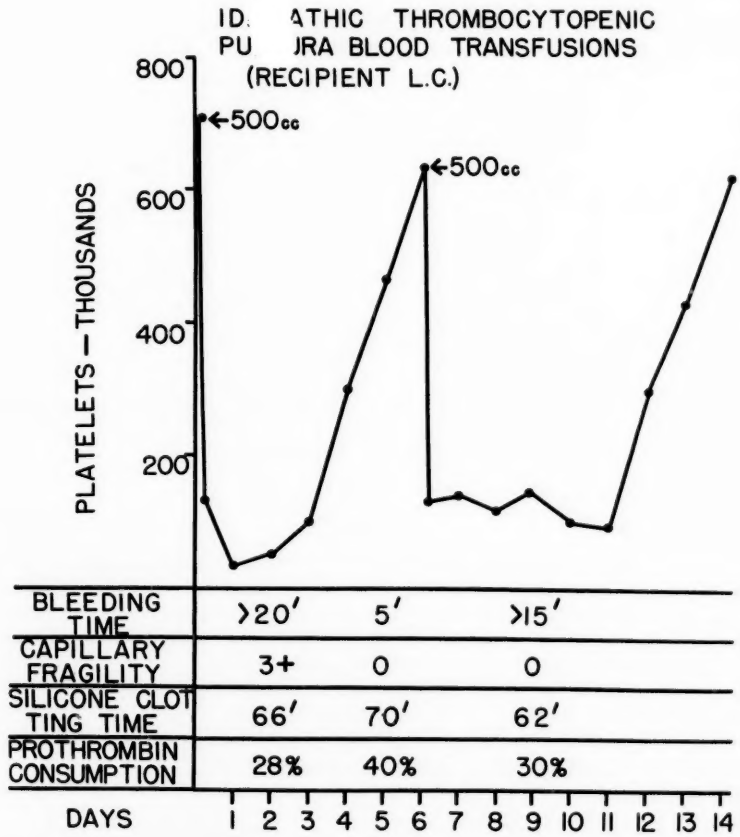

Fig. 3.—Changes produced in platelets, bleeding time, capillary fragility, and prothrombin consumption in a recipient who received successive transfusions of blood from two patients with idiopathic thrombocytopenic purpura. The figure illustrates the ability to produce, in a normal subject, the typical abnormal laboratory manifestations of this disease: thrombocytopenia, prolonged bleeding time, increased capillary fragility, and decreased prothrombin consumption. Deficient clot retraction was also observed.

studies on the survival of transfused platelets have indicated that the platelets disappear rapidly from the circulation in patients with idiopathic thrombocytopenic purpura.[14]

Plasma obtained from two of the donor patients (Nos. 2 and 9), after their platelet levels had returned to normal following splenectomy, caused approximately the same degree of thrombocytopenia in the recipients as it had before splenectomy. The observations on one of these subjects are charted in Fig. 4.

Moreover, transfusion of thrombocytopenic plasma into a patient whose spleen had been removed during gastrectomy for carcinoma of the stomach caused a definite drop in this subjects's platelet count (Fig. 6).

The effect of ACTH and cortisone on the circulating thrombocytopenic factor was studied in two patients with thrombocytopenia after splenectomy. In one of these (No. 3, Table I), there had been only a transient rise in the platelet

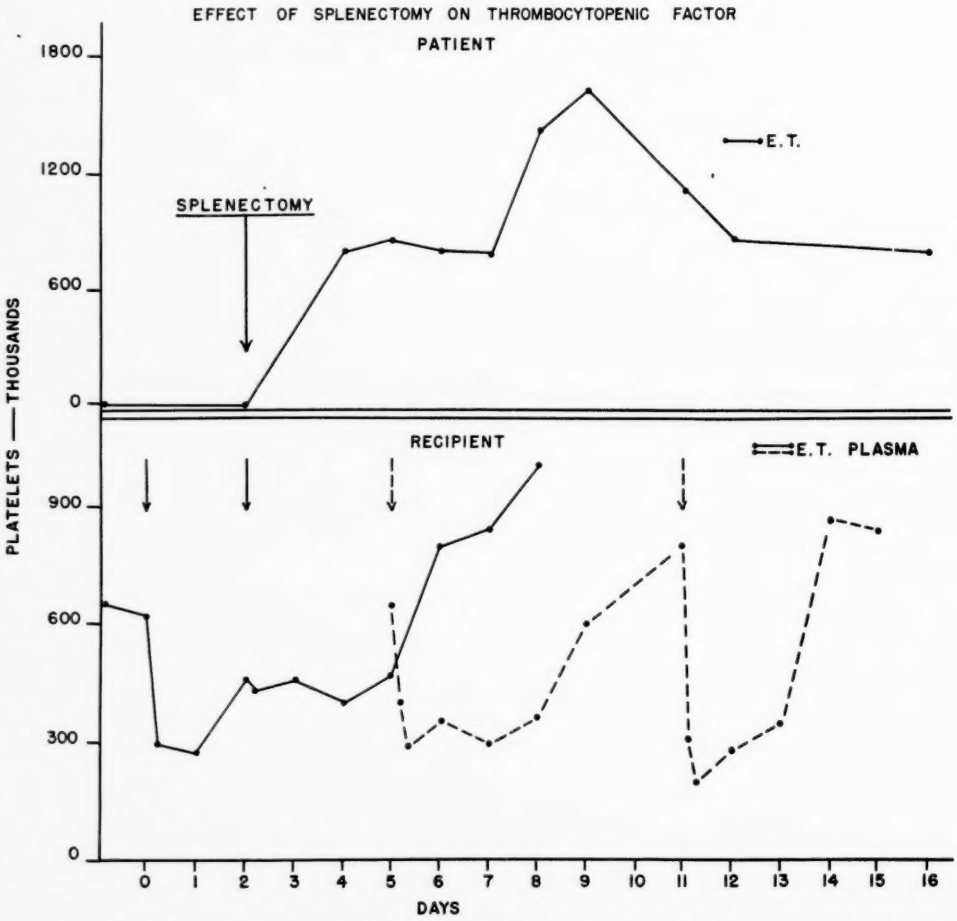

Fig. 4.—Failure of thrombocytopenic factor to disappear from the plasma after splenectomy. Solid lines in the bottom segment of the chart represent data for transfusion given to recipient prior to splenectomy; dotted lines represent similar data for blood obtained post-splenectomy after the patient's platelet count had become elevated above normal.

count following splenectomy three months previously, and hemorrhagic manifestations had persisted. Both ACTH and cortisone, given at different times, failed to influence either the number of platelets or the presence of the plasma thrombocytopenic factor. The second patient (No. 10) retained her thrombocytopenia for six or more months after her spleen had been removed in 1946; her platelets then slowly rose to normal values. She had remained asymptomatic

until petechiae again reappeared four days prior to the observations recorded in Fig. 5. Coincident with the administration of cortisone, the platelets increased to more than one million per cubic millimeter, and her plasma lost its ability to produce thrombocytopenia when transfused into the same recipient who had been used prior to the cortisone therapy.

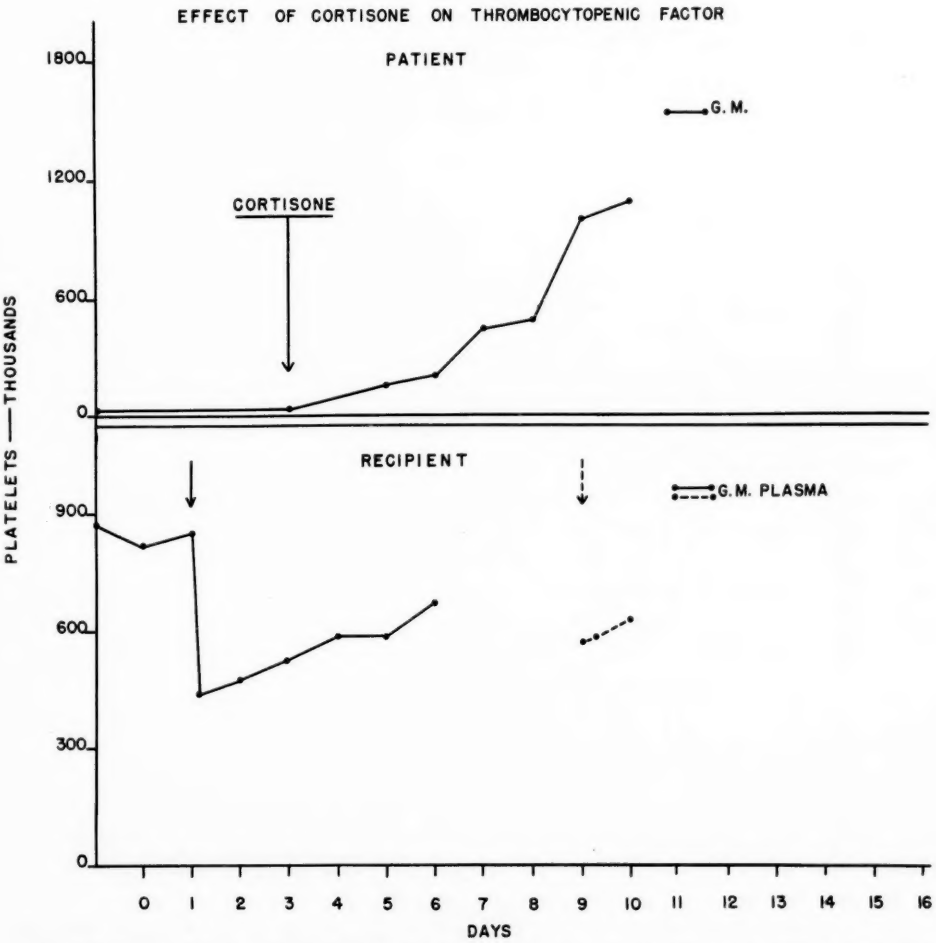

Fig. 5.—Effect of cortisone on platelet count and thrombocytopenic factor in a young woman who had developed a recurrence of thrombocytopenia four years after splenectomy. Three hundred milligrams of cortisone were injected daily for three days, and 200 milligrams daily for five additional days. The solid lines in the lower segment of the chart illustrate the platelet reduction produced in a recipient by administration of plasma obtained from this patient prior to cortisone therapy. The broken lines illustrate the failure of plasma obtained after the platelet count had returned to high levels to cause thrombocytopenia in the same recipient. These latter observations could be continued for only twenty-four hours because the subject was discharged from the hospital. However, in the forty-one tranfusion studies made to date, the thrombocytopenia invariably occurred within this period of time, or failed to develop at all.

Initial studies on the nature of the thrombocytopenic factor show that it is a plasma constituent present in the globulin fraction*[15] and is stable in acid citrate dextrose solution for periods of at least nine days at minus 5, plus 5, and plus 25° C. (Fig. 6). An attempt is being made to develop in vitro methods for demonstrating the thrombocytopenic factor.

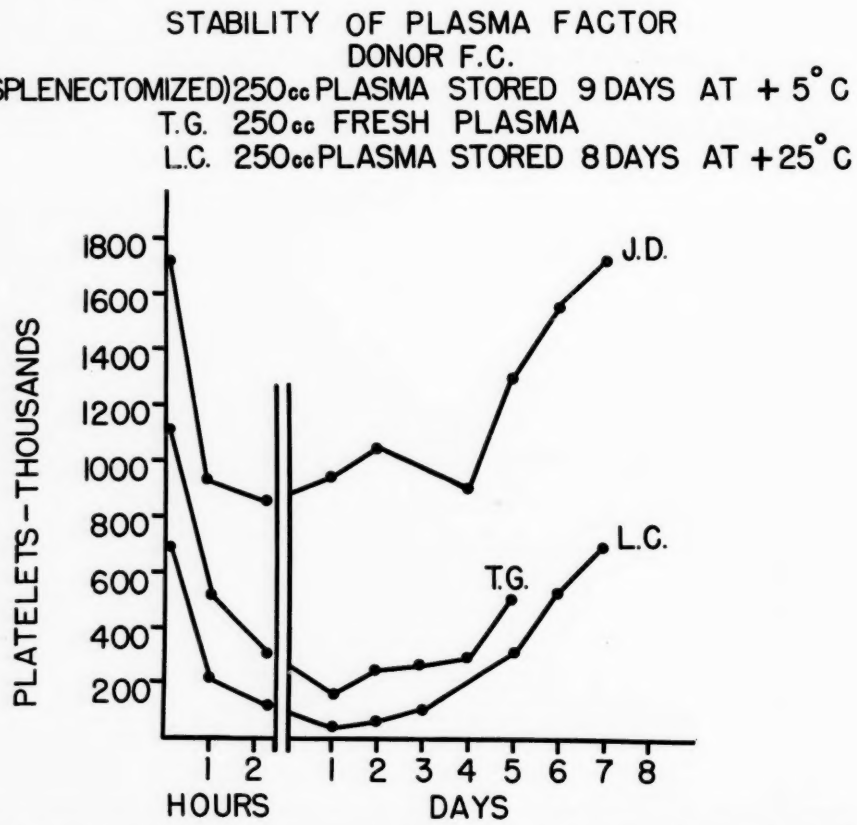

Fig. 6.—The stability of the thrombocytopenic factor in acid citrate dextrose plasma stored at different temperatures. Transfusions were given at "0" time. No definite loss of activity was noted under these conditions of storage. Of interest is the effect of the plasma on a recipient splenectomized for carcinoma of the stomach. A definite drop in the platelet count occurred (recipient J. D.).

DISCUSSION

The total implication of these results is not as yet apparent. It is clear, however, that many patients with idiopathic thrombocytopenic purpura have a relatively stable, circulating thrombocytopenic factor, presumably in the globulin fraction of plasma. When the platelet count rises after splenectomy, the factor does not necessarily disappear from the plasma. On the other hand, in the one patient whose platelet level did return to normal during therapy with cortisone, the thrombocytopenic plasma substance was no longer demonstrable. It is pos-

*We are indebted to Doctor Reubenia Dubach for performing this fractionation. Method X^{15} was carried through Step No. 1.

sible that the frequent relief of thrombocytopenia by splenectomy is due in part to a decrease in the rate of platelet destruction sufficient to permit the marrow to compensate for the platelet-removing effect of the thrombocytopenic factor. Much more experience will be needed before all possibilities can be adequately evaluated. Idiopathic thrombocytopenic purpura may not be a single entity, but a syndrome in which more than one pathogenic mechanism may be involved. The role of the spleen, and of the spleen-bone marrow interrelationship still lacks precise definition.

It is worth noting that the two patients with idiopathic thrombocytopenic purpura and the two with secondary thrombocytopenia in whom the platelet-reducing factor could not be demonstrated all failed to respond to splenectomy. Experience is too limited to evaluate this result. Furthermore, the presence of the thrombocytopenic factor could not be correlated with the occurrence of remission following splenectomy.

SUMMARY

A thrombocytopenic factor has been demonstrated in the whole blood and plasma from eight of ten patients with idiopathic thrombocytopenic purpura and from one of three subjects with secondary thrombocytopenia. Administration of 500 c.c. of citrated whole blood or its plasma equivalent from these nine patients caused a prompt, and often dramatic decrease in the platelet counts of nonthrombocytopenic recipients. The thrombocytopenic effect persisted for five to seven days and, when severe, was associated with a prolonged bleeding time and decreased prothrombin consumption. The factor was stable for at least nine days at 5° C, and for at least eight days at 25° C. In the one plasma fractionation that has so far been done, the thrombocytopenic factor was found in the globulin fraction. Two patients who were demonstrated to have the platelet-reducing factor prior to splenectomy still had the factor present after platelet counts had returned to normal following removal of the spleen.

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ENZYME STUDIES ON HUMAN BLOOD

XI. The Isolation and Characterization of Thromboplastic Cell and Plasma Components

George Y. Shinowara, Ph.D.
Columbus, Ohio

RECENT investigations on the blood coagulation mechanism have centered largely on the problem of the thromboplastic activation of prothrombin. Concepts concerning this reaction are numerous, but they can be classified generally into two categories. First, there are those based on the premise that thromboplastic activity exists in circulating blood as a zymogen. Thus Quick and Stefanini[22, 23, 24, 31] are of the opinion that plasma thromboplastinogen becomes activated by platelet thromboplastinogenase into active thromboplastin, which together with calcium converts prothrombin to thrombin. Milstone,[17, 19] on the basis of three phase experiments, concluded that the precursor complex found in plasma euglobulin becomes converted to thrombokinase (thromboplastin) by an autocatalytic process in the presence of calcium. This zymogen concept was formulated as early as 1912 by Collingwood and MacMahon,[9] who suggested that an inactive form of thromboplastin might be contained in platelets. Several years later Bordet[2] and Dale and Walpole[10] recognized the existence of such a precursor in plasma.

In the second group are those theories which have as their basis the active form of thromboplastin rather than its precursor. Brinkhous[4] takes the view that thromboplastin, per se, exists in platelets, and that it is liberated by plasma thrombocytolysin. Seegers and his group[25, 33] also retain the classical concept that thromboplastin and calcium activate prothrombin but have presented evidence that this activation is made more rapid by plasma Ac-globulin and by a platelet substance with a similar activity. Ac-globulin is probably identical to Factor V of Owren,[20] accelerator factor of Fantl and Nance,[12] and the labile or plasmatic co-factor of Quick[21] and Honorato.[15] Chargaff and West[7] centrifuged plasma at 31,000 ×g. and obtained a minute amount of reddish-brown pellets consisting of cellular debris and thromboplastic protein similar in activity to that of tissue cells.

Presented in this communication are quantitative experiments on the isolation and characterization of two substances from normal human blood, thromboplastic cell component (TCC), and thromboplastic plasma component (TPC). They differ greatly in chemical and physical properties as well as in source. However, they have similar biologic effect: *together, but not separately,* they behave like tissue thromboplastin in respect to the extent and the rate of pro-

From the Department of Pathology, The Ohio State University, College of Medicine.
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thrombin activation. The data moreover suggest a stoechiometric relationship between the two substances rather than that one is an enzymatic precursor and the other an activator. Therefore the evidence presented here is not compatible with either the active enzyme or the zymogen-kinase concepts of blood thromboplastin activity.

One unit of TCC is defined here as the minimum required in the presence of a known excess of TPC and with optimum calcium concentration to convert 1 unit prothrombin into 1 unit thrombin within thirty minutes, at pH 7.2, at 25° to 28° C. The unit of TPC is similarly defined. Therefore these units have as their final basis the unit of thrombin as does the unit of prothrombin and of tissue thromboplastin, all of which have been defined previously.[26]

METHODS AND MATERIALS

Collection of Blood.—Approximately 15 ml. of blood were obtained by venipuncture with a syringe containing 1.5 ml. of 4 per cent sodium citrate solution. A definite volume of the citrated blood was removed for the determination of final phase coagulation time. The remainder was centrifuged in calibrated 15 ml. graduated tubes at 1° C., 1,650 ×g., for thirty minutes. The cell and total volumes were recorded and the plasma was pipetted off. The cells and plasma were processed immediately or stored at -25° C.

Final Phase Coagulation Time.—To 0.8 ml. of whole citrated blood was added 0.2 ml. of standard human thrombin 5 U. per milliliter,[26] and the clotting time was determined. Another clotting time was determined by the same technic except that the standard thrombin reagent also contained 0.2 mg. protamine sulfate per milliliter. In seventy-five normal individuals the average clotting time with thrombin alone was 16.9 seconds with a range of 13.9 to 22.0. The clotting time with protamine thrombin reagent averaged 0.7 of a second faster. In over 300 patients significant differences between the final phase clotting times with and without protamine sulfate were found only in those who had received therapeutic heparin.

Fractionation of Plasma.—Fraction I sediment was obtained from 4 ml. of plasma and was dissolved in 4 ml. citrate-phosphate buffer as previously described.[26, 28] The alcohol-plasma supernatant was diluted and the prothrombin fraction precipitated out at pH 5.05.[26]

Determination of Prothrombin.—The activity of the prothrombin fraction obtained as described was determined by the homologous isolation procedure.[26] The mean value found in fifty-one normal individuals was 81.8 U. per milliliter, S.D. ± 14.5.[27]

Determination of Fibrinogen.—The thrombin-clottable protein of Fraction I solution obtained was determined by the chemical technique.[28] The mean plasma fibrinogen level in fifty-nine normal individuals was 0.26 per cent, S.D. ± 0.05.[28]

Isolation of TPC.—Two milliliters of Fraction I solution were heated in a water bath at 56° C. for five minutes. The resulting sediment was removed by centrifugation in Lusteroid tubes at 32,000 ×g., 1° C., for thirty minutes. The supernatant solution (Fraction I—NC) was tested for TPC activity as described below.

Isolation of TCC.—The technique used was similar to that for tissue thromboplastin.[26] When not in use the glass bowl of the Waring Blendor was placed in a -25° C. refrigerator and the temperature of the citrate-phosphate buffer (pH 7.2; Γ/2, 0.129) and the centrifuge (International PRI) was between 0° and 1° C. throughout the following procedure. Between 5 and 10 ml. of the packed erythrocytes, leucocytes, and platelets obtained in the initial centrifugation of whole blood were made up to 80 ml. with buffer, blended for approximately two minutes, and then centrifuged in Lusteroid tubes in an angle head at 32,000 ×g. for thirty minutes. The supernatant fluid was decanted off and the sediment was blended and centrifuged as before. This last step was repeated two to four additional times or until the supernatant fluid contained less than 5 mg. per cent protein by the sulfosalicylic acid turbidity method.[16] The final sediment was lyophilized: initial temperature, -25° C., and

pressure, 30 micra mercury. The resulting *crude TCC powder* was weighed and stored over silica gel in a vacuum desiccator. *Stock TCC solution* was prepared on the day of an experiment as follows. A 0.2 per cent suspension of the crude powder in citrate-phosphate buffer was stirred and heated at 56° C. for five minutes and then allowed to sediment for at least fifteen minutes. The fluid was pipetted off and filtered through two layers of cotton filamented gauze.

Determination of TPC Activity.—Into each of about twelve tubes was pipetted 0.5 ml. of diluted pooled normal prothrombin fraction, 20 U. per milliliter, and 0.5 ml. of TCC reagent solution, approximately 60 to 70 U. per milliliter. To one (reagent control) was added 0.5 ml. citrate-phosphate buffer; and to the other tubes, 0.5 ml. varying dilutions of the Fraction I—NC solution. The addition of 0.5 ml. 0.025M calcium chloride solution to each of the foregoing mixtures at 25° to 28° C. began the activation time. Thrombin was determined[26] on 0.2 ml. aliquots at least twice during the last five minutes of the thirty-minute activation period. The highest dilution of the Fraction I—NC solution resulting in the complete conversion of prothrombin to thrombin was considered the end point. The TPC control activation mixture consisted of the following: prothrombin, Fraction I—NC at the end point dilution, and calcium. The unit thrombin per 0.2 ml. test activation mixture was determined from the standard thrombin clotting time curve previously established.[26] However, the thrombin titers of both the TPC and the TCC controls were usually so low that the following equation was employed when the clotting time (C.T.) was over 70 seconds:

$$\text{Units thrombin} = 1/\text{C.T.} \times 0.07.$$

The *net* TPC activity in units per 0.2 ml. activation mixture was calculated by subtracting from the unit thrombin of the test mixture the sum of the thrombin units of the reagent TCC control and the TPC control. The TPC units per milliliter plasma were then calculated from the *net* activity employing the equation as already described for prothrombin.[26]

Determination of TCC Activity.—Various dilutions of the stock TCC solution were tested with a procedure identical to the foregoing. The reagent TPC was prepared as follows. A 1 per cent suspension of Fraction I[8] obtained from pooled normal dried plasma was heated for five minutes at 56° C. and then centrifuged at 32,000 ×g. and 1° C. The supernatant solution, Fraction I—NC, was diluted to 60 to 70 U. per milliliter TCC and reagent TPC controls were determined in every case. From the *net* activity per 0.2 ml. activation mixture was determined the units per milliliter of stock TCC 0.2 per cent cell sediment solution. Employing the basic equation used for TPC, the unit TCC per milliliter whole blood was calculated from the net activity per 0.2 ml. activation mixture, the known weight yield of the crude cell powder, the volume of the cells processed, and the hematocrit corrected for the citrate anticoagulant dilution. For both TPC and TCC determinations, another control activation mixture was set up as follows: 0.5 ml. prothrombin, 20 U. per milliliter; 0.5 ml. placental thromboplastin, 50 U. per milliliter; 0.5 ml. buffer; and 0.5 ml. 0.025M calcium chloride. All the test and reagent solutions except calcium chloride were made up in citrate-phosphate buffer.[26]

BIOLOGIC ACTIVITY

Fig. 1 presents data on the effect of concentration of blood cell reagent (TCC) and Fraction I—NC (TPC), together and separately, on prothrombin activation. The stock TPC and TCC reagents were prepared and tested as described in Methods and Materials. In all the activation mixtures in these experiments the prothrombin concentration was 5 units per milliliter. Within fifteen to thirty minutes' activation time 0.2 ml. of a control mixture containing placental thromboplastin clotted 0.8 ml. standard fibrinogen reagent in fifteen seconds. Therefore there was complete conversion since such a clotting time represents 1.0 U. per 0.2 ml. or 5 units thrombin per milliliter activation mixture.[26] It is shown in *Curve 1* that 0.012 mg. of the cell reagent alone can

convert a maximum of 7 per cent of the prothrombin to thrombin. However, the same amount plus 1.2 mg. Fraction I—NC., which alone had a conversion capacity of at the most 20 per cent, resulted in the complete conversion of prothrombin. In *Curve 3* the low conversion capacity of Fraction I—NC alone at various concentrations is demonstrated. With 0.6 mg. Fraction I—NC alone there is only 10 per cent conversion, but together with 0.025 mg. cell reagent there is 100 per cent conversion. Fraction I—NC and blood cell reagent separately in concentrations higher than those shown in Fig. 1 did not result in the complete conversion of prothrombin. Moreover, in all cases the net activity of either TPC or TCC was far in excess of the sum of those of the test and reagent controls.

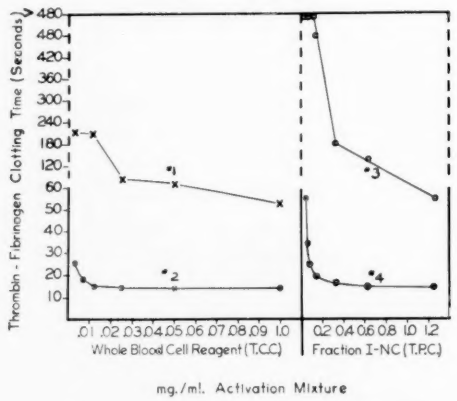

Fig. 1.—Prothrombin activation by whole blood cell reagent and Fraction I—N.C. reagent alone and together at varying concentrations. Γ/2, 0.129; pH, 7.2; activation time, 30 minutes; 26° C.; prothrombin, 5 U. per milliliter; calcium, 0.0062M.

Curve 1, Cell reagent alone. *Curve 2*, Cell reagent plus 1.2 mg. Fraction I—N.C. per milliliter activation mixture. *Curve 3*, Fraction I—N.C. alone. *Curve 4*, Fraction I—N.C. plus 0.025 mg. cell reagent per milliliter activation mixture.

In Fig. 2 are the activation rates of 1.25 mg. Fraction I—NC and 0.05 mg. cell reagent together, *Curve 3*, in which complete conversion is evident after fifteen minutes activation. At this time, with Fraction I—NC alone, *Curve 1*, there is no conversion, and with cell reagent alone, *Curve 2*, approximately 6 per cent. The similarity in the rate and extent of prothrombin conversion of the placental thromboplastin control, *Curve 4*, to the combined effect of TPC and TCC, *Curve 3*, is also noted.

Fig. 3 presents data on three-stage experiments. Three identical mixtures were made up as follows: 0.50 ml. TCC dilution, 20 U. per milliliter; 0.50 ml. TPC solution, 20 U. per milliliter; 0.25 ml. buffer; and 0.50 ml. 0.025M calcium chloride. Prothrombin, 0.25 ml., 34.4 U. per milliliter, was pipetted immediately after the calcium addition to the first tube; thirty minutes after to the second tube; and sixty minutes after to the third tube. The prior activation of TCC and TPC in the presence of calcium did not result in any acceleration of the prothrombin conversion rate. Identical results were obtained in similar three-stage experiments in which calcium was added at varying intervals to TPC, TCC, and prothrombin mixtures.

In Table I are recorded data on twenty-one blood samples from thirteen individuals without clinical evidence of hypo- or hypercoagulative states. The final phase coagulation times on twelve samples were obtained with standard thrombin reagent and were within one second of the coagulation times with the thrombin-protamine reagent. Prothrombin and fibrinogen levels were obtained on all twenty-one samples.

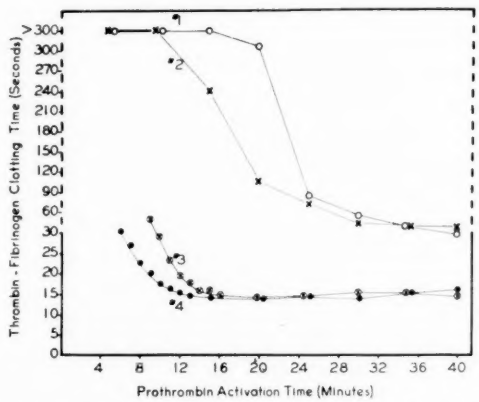

Fig. 2.—The prothrombin activation by TCC and TPC, separately and together, compared with that by placental thromboplastin. In all activation mixtures: Γ/2, 0.129; pH, 7.2; 26° C.; prothrombin, 5 U. per milliliter; calcium, 0.0062M.

Concentration per milliliter activation mixtures: *Curve 1*, 1.25 mg. Fraction I—NC alone (TPC). *Curve 2*, 0.05 mg. cell reagent alone (TCC). *Curve 3*, 1.25 mg. Fraction I—NC and 0.05 mg. cell reagent. *Curve 4*, 12.5 U. placental thromboplastin.

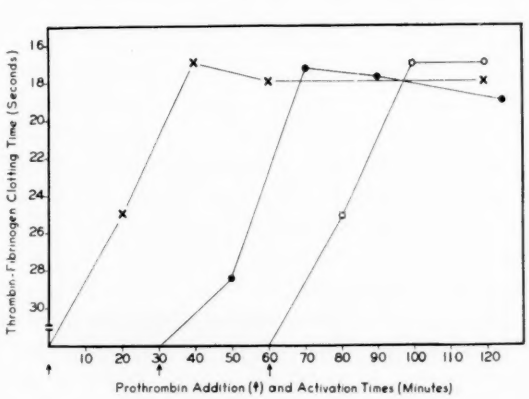

Fig. 3.—Results of attempts to activate TPC and TCC for 0, 30, and 60 minutes prior to the addition of prothrombin. Concentrations in final activation mixtures: Prothrombin, 4.3 U. per milliliter; TCC, 5 U. per milliliter; TPC, 5 U. per milliliter; calcium, 0.0062M. Γ/2, 0.129; pH, 7.2; 26° C.

The thromboplastic plasma component activity in units per milliliter of plasma varied widely between individuals. The average was 97.5 U. per milliliter with a range of 17.1 to 273.5. More than one determination was done on four individuals; in one, Case 590, extremely variable results were obtained. TPC activity was also determined on both the 1,600 ×g. and the 32,000 ×g. plasma

TABLE I. THROMBOPLASTIC CELL AND PLASMA COMPONENTS, AND OTHER COAGULATION STUDIES ON THIRTEEN INDIVIDUALS

CASE	AGE AND SEX	DATE	FINAL PHASE COAGULATION TIME (SEC.)	PLASMA PROTHROMBIN (U./ML.)	PLASMA FIBRINOGEN (%)	THROMBOPLASTIC	
						CELL COMPONENT (U./ML.)*	PLASMA COMPONENT (U./ML.)
641	23 F	3-16	16.50	63.1	0.251	242.0	——
635	23 F	3-13	17.55	62.8	0.254	——	24.7
		4-10	——	70.0	0.291	91.6	——
		6-21	16.40	81.6	0.261	——	79.5
642	23 F	3-16	——	64.0	0.172	207.6	29.8
		8-2	16.00	75.0	0.128	704.1	31.6
721	25 M	5-4	——	86.1	0.252	——	59.1
623	25 M	3-8	17.30	101.5	0.214	——	40.9
670	25 M	4-4	——	107.9	0.226	——	59.5
534	26 F	2-2	18.60	100.9	0.464	228.1	——
756	26 M	5-17	——	71.0	0.189	1,930.0	105.2
		5-22	14.85	67.3	0.184	152.9	——
723	30 M	5-8	——	81.5	0.156	885.0	125.5
669	30 M	4-4	——	90.1	0.226	290.3	134.6
		4-13	18.80	84.4	0.484	——	128.2
		6-21	14.75	116.0	0.303	——	80.5
600	31 M	2-28	22.20	100.7	0.387	190.0	——
557	34 M	2-10	18.10	109.0	0.372	608.5	——
590	36 M	2-23	——	82.5	0.345	——	273.0
		6-1	——	98.5	0.377	1,504.0	273.5
		8-14	15.50	110.3	0.298	499.5	17.1

*Whole blood.

obtained from three individuals. In one, identical results were obtained, and in the other two the activity of the 32,000 ×g. plasma was slightly higher than the 1,600 ×g. plasma.

The thromboplastic cell component activity was generally high in all the thirteen samples tested, indicating a tremendous excess of this coagulation component in human blood. However, the variations between samples were even greater than those of TPC. The range in TCC activity was 91 to 1,930 with the average of 579 U. per milliliter whole blood. There was no correlation between the platelet counts, which were within the normal range, and the TCC activity. The average weight yield of the dry TCC powder was 4.5 mg. per milliliter whole blood or 10.0 mg. per milliliter whole cells. In the preparation of the stock TCC solution (see Methods and Materials) it was found that 60 to 70 per cent of the dried powder goes into solution. In more recent experiments considerably higher TCC activity than that reported here has been found in normal blood cells. This has been attributed primarily to a more efficient extraction technique.

The question may arise as to why whole blood cells rather than platelets were used as the source. It has been the experience of this laboratory that a cell fraction rich in morphologically intact platelets, and almost devoid of erythrocytes and leucocytes, is readily obtained by differential centrifugation. However, the quantitative yield is unsatisfactory as evidenced by the consistent finding of a variable but significant number of platelets in the buffy coat and the so-called red cell layer. Furthermore, it was the intention in the present study to obtain a product with minimum preliminary handling of cells. Nevertheless, experiments on the TCC activity of various cell fractions have been under way

in this laboratory. Erythrocyte, leucocyte, and platelet counts are all done on every cell fraction, as well as morphologic observations of the platelets particularly, by a phase microscope on vital preparations. Results of such experiments[30] thus far have indicated that TCC activity is found in extracts of not only platelet but also of platelet-poor and leucocyte-poor erythrocyte fractions. Another attack on this problem bearing on the possible association of platelets with TCC activity was made by analysis of whole blood cells obtained from individuals with low counts. The platelet count, diagnosis, and the TCC activity of three such patients studied thus far were as follows: 30,800 per cubic millimeter, Hodgkin's disease, 1,623 U. milliliter; none seen, Hodgkin's disease, 1,072 U. per milliliter; and, none seen, myeloid leucemia, 87 U. per milliliter. The actual number of platelets per approximately 2,000 red cells was counted, employing Dameshek's technique.[11] The TCC activity was within the range of that found in the normal group. This finding is compatible with the frequent observation that individuals with very low platelet counts have normal gross clotting times.

CHEMICAL AND PHYSICAL PROPERTIES

It has been demonstrated already that the TCC activity is in the 32,000 ×g. sediment obtained by the centrifugation of lysed blood cells, whereas the TPC activity is found in the nonclottable subfraction of plasma Fraction I, and it remains in solution after prolonged centrifugation at 32,000 ×g. It is also evident that both TPC and TCC are stable at 56° C. Heat was employed to remove fibrinogen from Fraction I rather than to accomplish this by the addition of another coagulation component, i.e. thrombin.

The primary objective in this study was to obtain TCC and TPC free of other coagulation factors, i.e. biologic rather than chemical purity. Nevertheless, additional analyses were made for comparative purposes on the solids in the stock TCC and TPC solutions as well as on those in the stock placental tissue thromboplastin solution.[26] From 100 to 120 ml. of these solutions were prepared from pooled blood cell powder, Fraction I from normal dried human plasma, and crude thromboplastin powder.[26] For the solid content and the lipid determinations an aliquot of the resulting solutions was lyophilized, initial temperature –25° C. and pressure 30 micra of mercury. The resulting powders, which were all white, were weighed and the yields corrected for the salt content.

The activities of the foregoing preparations are recorded in units per milligram soluble protein in Table II. Each of the lyophilized powders dissolved readily and completely in approximately 6 ml. of water in the following amounts: TPC, 223 mg.; TCC, 70 mg.; and placental thromboplastin, 67 milligrams. Each of the aqueous solutions was added slowly with mixing to approximately 150 ml. of Bloor solvent. The mixture was brought to boil, allowed to stand for twenty-four hours, filtered, and then the volume was made up to 200 ml. with the Bloor solvent washings of the precipitate. The results of the differential lipid analysis done by the Boyd modification[3] of the Bloor micromethod[1] on the extracts are recorded in Table II. The high lipid contents, particularly phospholipids, of both TCC and thromboplastin are noted. Chargaff, Bendich, and Cohen[5] reported between 38.8 and 49.8 per cent total lipids in highly purified

TABLE II. THE RESULTS OF CHEMICAL STUDIES ON THROMBOPLASTIC CELL AND PLASMA COMPONENTS COMPARED WITH THOSE ON TISSUE THROMBOPLASTIN

COAGULATION FACTOR	BIOLOGIC ACTIVITY (U./MG.)	LIPIDS (GM./100 GM. SOLID)				TYROSINE EQUIVALENT	REACTION WITH SULFOSALICYLIC ACID $K_{450} = \frac{D}{C}$
		NEUTRAL	CHOLESTEROL		PHOSPHOLIPIDS		
			FREE	ESTER			
Thromboplastic plasma component	18.4	1.37	0.82	0.36	0.99	15.96	6.72
Thromboplastic cell component	180.0	5.82	4.31	0.00	9.71	42.81	2.17
Placental tissue thromboplastin	158.7	7.60	2.51	0.19	13.34	45.78	2.94

thromboplastin prepared from beef lung. Therefore, the human thromboplastin, which was prepared for reagent and control purposes and not for chemical purity, is approximately 50 per cent pure on the basis of lipid analysis.

The total tyrosine equivalents of the TPC, TCC, and placental thromboplastin solutions were determined with phenol reagent.[13, 14] The Coleman Junior spectrophotometer, at 650 mμ, was the instrument employed. The results in terms of grams equivalent to 1 Gm. chemically pure tyrosine in Table II indicate the low concentration of tyrosine in TCC and thromboplastin. The latter is compatible with their high lipid content.

The TPC, TCC, and placental thromboplastin solutions were reacted with sulfosalicylic acid.[16] The optical density at 450 mμ was determined with a Coleman Junior spectrophotometer. The results in Table II indicate the chemical similarity of TCC and placental thromboplastin, a finding also shown by lipid and tyrosine analysis. The mean K factor for pure human plasma albumin, in six determinations, was found to be 14.95, S.D. ± 0.53.

Ultraviolet absorption studies on TPC, TCC, and placental thromboplastin solutions were done in a quartz spectrophotometer (Beckman, Model D.U. with ultraviolet accessory) at pH 7.2; Γ/2, 0.129; approximately 0.05 per cent protein. The TPC showed a distinct maximum extinction at 280 mμ, and minimum extinction at 254 mμ as previously reported[28] for the nonclottable subfraction of Fraction I. In contrast, TCC and placental thromboplastin both have a minimum extinction at 250 mμ and a gradually rising plateau-like maximum extinction at approximately 270 mμ for the former and 260 mμ for the latter.

DISCUSSION

The isolation in a native state from human blood alone of all the biologic components absolutely necessary for coagulation has been the primary objective of this laboratory. This objective was realized by the preparation from whole blood cells of a stable powder which resembles tissue thromboplastin in chemical and physical properties but not in biologic activity. Tissue thromboplastin is a lipoprotein, as has been clearly demonstrated by Chargaff[5, 6] which completely

converts prothrombin in the presence of calcium ion alone.[25] On the other hand, the blood cell powder, tentatively named thromboplastic cell component (TCC), is also a lipoprotein but requires a plasma component (TPC) as well as calcium to activate prothrombin. TCC must be adsorbed on or be within certain blood cells for the following reasons. The activity occurs in washed or unwashed intact normal blood cells sedimented at or below 1,600 ×g., but after the cells have been homogenized it is found only in a high gravity sediment. TCC is associated not only with platelets, but also with other blood cells, as shown by experiments in which high activity was found in extracts of platelet, and of platelet-poor, cell fractions obtained by differential centrifugation of normal blood and in whole blood cell preparations from individuals with low platelet counts.

Thromboplastin, per se, does not exist in human blood cells, for the high gravity sediment in great excess with calcium alone does not quantitatively convert prothrombin to thrombin. In all the determinations of TCC activity there was very little or no thrombin formed in the control tube, i.e. in the activation mixture without reagent TPC. In another series of experiments[30] samples for study were obtained as follows: after approximately 500 ml. had been collected, a syringe was substituted for the donor tube. On all such samples from several individuals no thromboplastin lipoprotein could be found in the cells or plasma. Furthermore, it is improbable that the slight but never complete conversion of prothrombin by TCC and TPC separately can represent traces of thromboplastin activity. Theoretically, the experimental conditions in this study could result in higher rather than lower thrombin formation by the controls: for example, silicone-coated needles and glassware were not used to further minimize cellular disintegration.

The biologic activity of TPC cannot replace that of TCC for each requires the other for prothrombin conversion. TPC has no similarity to tissue thromboplastin in any respect and differs greatly from TCC in chemical and physical properties. The source of TPC activity, the nonclottable globulins of Fraction I, suggests its possible identity with the antihemophilic globulin.[8, 32] In the next communication[29] it is demonstrated by the determination of TPC in the blood plasma of known hemophiliacs that this presumption is probably correct. However, it is difficult to relate TPC to Factor V,[20] Ac-globulin,[33] labile factor,[21] and others which have been reported to accelerate the prothrombin-thromboplastin reaction. In a previous communication[26] it was shown that diluted fresh plasma does not increase the rate or extent of this reaction. Subsequently almost 1,000 prothrombin determinations have been done by the homologous isolation procedure. The addition of dilute pooled fresh plasma or Fraction I—NC did not increase in any way the reaction of tissue thromboplastin with the prothrombin fraction. Thus the results of this laboratory cannot assign TPC as a co-factor or an accelerator of thromboplastin and calcium. Presumably, TPC could simulate the activity of labile factor provided that TCC is present as a contaminant in a prothrombin activation mixture containing a subminimal tissue thromboplastin concentration.

The reactions of the biochemical components, all isolated from human blood alone, can be summarized as follows:

$$\text{Prothrombin} + \begin{matrix}\text{Thromboplastic Cell Component} \\ + \\ \text{Thromboplastic Plasma Component}\end{matrix} + \text{Calcium} \rightarrow \text{Thrombin}$$

$$\text{Fibrinogen} + \text{Thrombin} \rightarrow \text{Fibrin}$$

In this scheme thrombin is formed only when both TPC and TCC react with prothrombin. By the same token normal blood remains fluid as long as the TCC is not released by the cells. (However, if there is tissue injury with the release of thromboplastin, prothrombin can be activated by both this substance and TCC plus TPC.)

The foregoing mechanism is in two rather than in three stages for several reasons. The present data demonstrate a stoechiometric, rather than a kinase-zymogen, relation between TPC and TCC. Moreover, the activation of prothrombin by TCC plus TPC is similar in rate and in extent to that by tissue thromboplastin. Finally, the prior activation of TPC and TCC with or without calcium did not increase prothrombin activation. In this conjunction it is interesting to note that in 1948 Milstone[18] demonstrated that washed rabbit platelet suspensions and calcium defibrinated euglobulin obtained from bovine plasma together but not separately activated prothrombin in two-stage experiments; erratic three-stage results did not permit him to make any conclusion that a thromboplastin zymogen was being activated. Nevertheless, despite such results, Milstone[17, 19] has retained the three-stage concept that prothrombokinase complex by autocatalytic or chain reaction becomes active thrombokinase which in turn activates prothrombin. Quick[23] and Stefanini[51] are also of the group favoring the plasma thromboplastin zymogen and the three-stage mechanism. The clotting mechanism, according to Seegers,[25] involves the active thromboplastin and is in four stages.

The concept in this communication is based only on the available direct evidence and is undoubtedly an oversimplification of the coagulation mechanism. Accessory and essential anti- and procoagulation factors may be isolated from blood and characterized. Therefore, the present concept involving blood components alone is considered only as a framework to which can be added the facts from future investigations.

SUMMARY

Thromboplastic cell component (TCC) and thromboplastic plasma component (TPC) *together but not separately* have an activity identical with that of tissue thromboplastin lipoprotein in the extent and in the rate of prothrombin conversion. The results indicate a stoechiometric rather than a kinase-zymogen relationship between these two blood components. TCC is a lipoprotein isolated by high gravity centrifugation of blood cell extracts. TPC is found in the nonclottable globulins of plasma Fraction I. Therefore, all the biochemical factors essential for coagulation can be obtained individually in measurable quantities from human blood alone. A concept of the coagulation mechanism not involving tissue thromboplastin is presented and discussed.

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ENZYME STUDIES ON HUMAN BLOOD

XII. Thromboplastic Plasma Component and Other Coagulation Factors in Hemophilia

George Y. Shinowara, Ph.D.
Columbus, Ohio

THE cause of the hypocoagulability of hemophilic blood has been attributed generally to the excess of anticoagulants or to the lack of a plasma factor. These divergent concepts have been reviewed by Pavlovsky[4] and by Quick,[5] respectively. The deficiency concept has been strengthened as the result of the extensive plasma fractionation program of Cohn and associates[2] in the Department of Physical Chemistry at the Harvard Medical School. The various fractions obtained from normal plasma were investigated for antihemophilic activity by the Thorndike Memorial Laboratory group.[3, 11] It was found that the nonclottable globulin of Fraction I is particularly active. Since the exact function of this globulin fraction in the coagulation mechanism is not known, it has been named antihemophilic globulin by clinical association. However, other investigators have postulated on the nature of the coagulation factor deficient in hemophilia. Thus Brinkhous[1] believes this to be plasma thrombocytolysin which releases the active thromboplastin enzyme from platelets and other formed elements of blood. On the other hand, Quick[5] concluded that this factor is the plasma thromboplastin zymogen which is activated by the platelet kinase.

In a previous communication of this laboratory[9] it was demonstrated that the nonclottable globulin of Fraction I and a lipoprotein of platelets and other blood cells *together but not separately* activate prothrombin. By identification and by association they were termed thromboplastic plasma component (TPC) and thromboplastic cell components (TCC), respectively. The present study includes the determination of these components and other coagulation factors in hemophilic blood.

SPECIMEN

Blood was collected as previously described[6] from twelve known hemophiliacs on the service of Dr. Charles A. Doan. Ten patients were in a clinically quiescent phase when each contributed blood for this study. The other two were hospitalized because of the acute exacerbation of their disease. Specimens were collected from these individuals at least two days after Fraction I, plasma, or whole blood therapy.

METHODS

The final phase coagulation times with and without protamine were determined on whole citrate blood. Fraction I sediment was obtained from 4 ml. plasma by the application of Cohn's low temperature principle[2] and reconstituted to the same volume. The plasma fibrinogen

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level and TPC activity were determined on this solution. The supernatant from the Fraction I separation was diluted and the prothrombin fraction was precipitated out at pH 5. TCC activity was determined on all the cells, including leucocytes, erythrocytes, and platelets, obtained by centrifuging whole blood at 1,600 ×g. The biochemical reagents were all obtained from human source and quantitatively standardized. These techniques and reagents have been described in detail.[6, 8, 9]

RESULTS

Table I presents thirty-six thrombin-fibrinogen clotting times on nine activation mixtures in which the constants were prothrombin, calcium, pH, and temperature. At various activation times, 0.2 ml. of the mixture was added

TABLE I. PROTHROMBIN ACTIVATION, WITH AND WITHOUT BLOOD CELL REAGENT, BY THE NONCLOTTABLE GLOBULIN OF FRACTION I FROM TWO HEMOPHILIC BROTHERS AND ONE NORMAL INDIVIDUAL

TUBE NO.	COMPONENTS PER ML. ACTIVATION MIXTURE* FRACTION I-NC (TPC) SOURCE	VOLUME (ML.)	CELL REAGENT (TCC) (U.)	THROMBIN FIBRINOGEN CLOTTING TIMES (SEC.) AT VARIOUS ACTIVATION TIMES 10 MIN.	20 MIN.	30 MIN.	40 MIN.
1	(Control)	0.0000	0.0	N	N	N	N
2†	(Control)	0.0000	0.0	33.5	17.1	14.4	14.6
3	(Control)	0.0000	12.0	N	620.0	94.8	85.0
4	Normal	0.0625	12.0	33.1	16.0	14.1	14.5
5	No. 669	0.0625	0.0	N	900.0	420.0	360.0
6	Hemophiliac	0.3750	12.0	900.0	56.2	35.0	27.9
7	No. 682	0.3750	0.0	N	N	2,100.0	1,800.0
8	Hemophiliac	0.3750	12.0	360.0	70.3	39.7	31.7
9	No. 692	0.3750	0.0	N	N	2,460.0	2,000.0

*Prothrombin, 5.2 U./ml.; calcium, 0.0062M; pH, 7.2; 27° C.
†Standard human placental thromboplastin, 5.2 U./ml., added.
N = No clot in 3,600 seconds.

to 0.8 ml. of standard fibrinogen reagent and the clotting times determined. Other mixtures containing varying concentrations of Fraction I—NC (nonclottable protein) were also tested. However, only data on mixtures with critical concentrations of Fraction I—NC are reported. In Tube No. 1, the absence of thrombin formation indicates no spontaneous prothrombin conversion with calcium alone. A minimum concentration of tissue thromboplastin was added in the activation mixture (Tube No. 2), resulting in the complete activation of prothrombin. Tube No. 3 represented the third reagent control and contained an excess of blood cell reagent: With TCC alone, there was at the most 15 per cent conversion of prothrombin as estimated from the 94.8 seconds clotting time after thirty minutes activation. Less than 3 per cent of the thrombin was obtained by 0.0625 ml. of normal Fraction I—NC (Tube No. 5); and less than 0.7 per cent by 0.375 ml. of both hemophilic Fraction I—NC (Tubes Nos. 7 and 9). However, when both normal Fraction I—NC and TCC were present in Tube No. 4, there was complete conversion as in the tissue thromboplastin control Tube No. 2. In other activation mixtures with greater concentrations of normal Fraction I—NC there was no increase in the extent of conversion at thirty minutes; with less, there was a diminution. Despite the fact that a *six times greater* volume of Fraction I—NC was present, the clotting times of hemo-

philic mixtures (Tubes Nos. 6 and 8) were 35.0 and 39.7 seconds, respectively, after thirty minutes activation in contrast to 14.1 seconds in the normal mixture No. 4. It is estimated that the stated volumes of hemophilic Fraction I—NC plus excess reagent TCC converted only 35 to 40 per cent of the prothrombin. Since there was incomplete conversion with maximum volumes of Fraction I—NC (Tubes Nos. 6 and 8) the unit of TPC activity originally defined[9] was necessarily revised. In such cases, the quantitative results are indicated in arbitrary units. Calculations from the basic data in Table I resulted in the following TPC activity per milliliter of plasma: normal No. 669, 134.6 units; hemophilic No. 682, 2.9 arbitrary units; and hemophilic No. 692, 2.3 arbitrary units.

The data on the twelve known hemophiliacs investigated thus far are presented in Table II. Final phase coagulation times with thrombin reagent on eight patients were all within normal limits. Determinations with protamine-thrombin reagent were also done and found to be within one second of those determined by thrombin reagent. Therefore, in these patients there was no anticoagulant of the type that inhibits the thrombin-fibrinogen reaction. There was no significant deviation from the normal in the prothrombin levels in all twelve individuals. The fibrinogen concentrations were generally normal except in three cases where there were moderate elevations. Determinations of TCC activity were done on four cases, and the results were definitely within normal limits. There were no qualitative differences in the TCC obtained from normal and hemophilic individuals as shown by reactions with Fraction I—NC.

Thromboplastic plasma component was determined in all twelve cases. The average was 3.8 arbitrary units as compared to 97.5 units found in the normal group.[9] The highest value of the hemophilic group was approximately one-half

TABLE II. THROMBOPLASTIC PLASMA COMPONENT AND OTHER DATA ON TWELVE HEMOPHILIACS

CASE NO.	AGE (YR.) AND SEX	FINAL PHASE COAGULATION TIME (SEC.)	PROTHROMBIN (U./ML.)	FIBRINOGEN (%)	THROMBOPLASTIC	
					CELL COMPONENT (U./ML.)	PLASMA COMPONENT (U./ML.)
665	1M	——	76.6	0.310	——	2.3A
836	2M	17.8	87.1	0.385	——	5.3A
779†	4M	17.1	67.7	0.180	——	9.6A
823	5M	16.7	63.6	0.207	——	5.1A
156	7M	——	58.2	0.176	——	4.7A
731	10M	——	51.0	0.194	——	0.7A
833	10M	21.6	62.9	0.305	——	1.4A
780	14M	18.3	92.8	0.369	861.8	4.0A
402	15M	18.4	61.0	0.179	146.8	4.7A
682*	19M	15.5	92.0	0.479	——	2.9A
692*	21M	——	68.4	0.325	1,572.5	2.3A
561†	31M	17.2	87.0	0.200	714.0	2.0A
Normal values	Mean	16.9	81.8	0.26	579	97.5
	Range or S.D.	13.9-22.0 (9)	S.D. ± 14.4 (7)	S.D. ± 0.05 (8)	91-1,930 (9)	17.1-273.5 (9)

A. Arbitrary units: The highest concentration of Fraction I—NC in activation mixtures did not completely convert prothrombin as required by the original definition[9] of the TPC unit.

*Case Nos. 682 and 692 are brothers. Other data in Table I.

†Case No. 561 is the maternal uncle of Case No. 779.

that of the lowest normal. However, of greater significance is the fact that hemophilic Fraction I—NC in maximum volumes did not completely convert prothrombin.

Each of the individuals had been previously diagnosed as classical hemophilia in one or more hematologic clinics. For example, the 21-year-old man (Case No. 692), had been first diagnosed at the age of 13 months. Subsequently, this has been confirmed several times, and as recently as two years ago. His younger and only brother (Case No. 682), who also had been repeatedly studied since infancy, was in the hospital when the blood for the present study was obtained. He had been admitted because of severe sublingual, gastrointestinal, and subcutaneous hemorrhage. The gross clotting time was eighty minutes. The diagnosis of hemophilia had been established in Case No. 561 at the age of 13 years. Subsequently, he has been hospitalized a number of times because of severe hemorrhages into joints and because frequent teeth extractions necessitated prophylactic and therapeutic treatment. The gross clotting times before transfusions were over sixty minutes. His sister's son (Case No. 779) was determined to be a hemophiliac in infancy because of the familial history, frequent spontaneous hemorrhages, particularly into the knee and elbow joints, and a slightly prolonged coagulation time. It is well known that despite definite familial history and classical symptoms markedly prolonged clotting times are not always demonstrated in young hemophiliacs, due to the difficulty in obtaining a satisfactory blood specimen.

DISCUSSION

The coagulation mechanism involving components isolated from blood alone was presented previously.[9] The present data indicate that the hypocoagulability of hemophilic blood is due to the marked deficiency of the thromboplastic plasma component.

The key to the experimental evidence leading to this concept of blood coagulation and of hemophilia was the isolation and the characterization of the thromboplastic cell component. Repeated attempts had been unsuccessful in this laboratory to increase the extent and the rate of the tissue thromboplastin activation of prothrombin with plasma and its derivatives. In this conjunction Fraction I particularly has been tested in such experiments because of its established effectiveness in lowering the gross clotting time when injected into hemophiliacs. The plasma prothrombin and placental thromboplastin lipoprotein employed in these experiments were of human origin and had been quantitatively standardized.[6] Equally unsuccessful were attempts to isolate from blood cells and plasma by high gravity centrifugation a substance with the biological activity of tissue thromboplastin.[10] However, in the high gravity sediment of blood cells was found a lipoprotein which not only clarified but also reconciled the negative results in the two apparently divergent experiments designed to further increase the prothrombin-tissue thromboplastin reaction and to isolate thromboplastin from blood. This blood cell lipoprotein and the nonclottable globulin of Fraction I, together but not separately, were found to be identical to tissue thromboplastin in respect to prothrombin activation. Therefore, the

thromboplastic cell component made possible investigations such as this, which could not be accomplished with tissue thromboplastin. The latter was used here only as a reagent control for prothrombin.

In the previous study[9] on normal blood it was stated that the thromboplastic plasma component is possibly identical to antihemophilic globulin,[3, 11] particularly because of their common source. The results here obviously indicate that the two are identical but primarily serve to explain the heretofore unknown function of the antihemophilic globulin in the coagulation mechanism. The results also confirm the findings of Brinkhous,[1] Quick,[5] and others that hemophilia is a plasma deficiency disease. However, the data here and in the previous communication are not compatible with the conclusion that the deficient factor is a thromboplastin zymogen[5] or a substance which releases active thromboplastin from platelets.[1]

SUMMARY

In all twelve known hemophiliacs the thromboplastic plasma component activity was definitely low in quantitative tests with prothrombin and thromboplastic cell component, standard reagents obtained from human blood alone. The function of this deficient factor in the coagulation mechanism is discussed.

The author is grateful to Mrs. Marian M. Starkey, B.S., and Mrs. Dolores M. Johnston, B.S., for their excellent technical assistance; to Dr. Charles A. Doan, Dean of the College of Medicine, for making his patients available for this study; and to Dr. Emmerich von Haam, Chairman of the Department of Pathology, for his valuable editorial suggestions.

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SPECTROPHOTOMETRIC STUDIES ON CLOT DENSITY

SAMUEL LOSNER, M.D., BRUNO W. VOLK, M.D., MENDEL JACOBI, M.D., AND STANLEY NEWHOUSE, B.A.
BROOKLYN, N. Y.

IN A previous report a photoelectric method of determining the prothrombin time was described.[1] It was observed that the light beam of the Coleman spectrophotometer (Junior Model 6A) moved to the left at the moment of clotting of the plasma and came to a standstill when the clot was formed. This phenomenon was explained by the increasing optical density and consequently decreasing transmittance of the plasma at the moment of clotting.

The impression was gained that a quantitative relationship existed between the change in optical density and the concentration of fibrinogen in the plasma. It was observed, furthermore, that while doing the prothrombin time with the spectrophotometer, the change in optical density for a given specimen of plasma was always constant when expressed in logarithmic terms as read from the (upper) scale of optical density. The latter is defined by:

$$D = - \log T$$

wherein D is the optical density and T the transmittance of the specimen of plasma.

While determining the prothrombin time spectrophotometrically in a series of dicumarolized patients with acute myocardial infarction, it was observed that the change in optical density at the moment of clotting went parallel with the sedimentation rate, and that with the approach of the latter to normal values the change in optical density decreased correspondingly.

The sedimentation rate being to a large extent subject to alterations in the fibrinogen content of the plasma,[2] this finding was believed to be further proof of the initial assumption that the change in optical density at the moment of clotting depended upon the fibrinogen concentration of the plasma. This change was termed *clot density* and defined as the difference of the optical densities before and after clotting multiplied by 100 while carrying out the one-stage Quick method of prothrombin determination with the Coleman spectrophotometer, as described previously, at the wave length of 500 mμ.[1]

The initial assumption, therefore, was formulated differently, namely, that the clot density varied with the fibrinogen concentration in the plasma. To prove the validity of this hypothesis a study was made of the clot densities of a series of solutions of human and bovine fibrinogen, as well as those of plasma specimens from normal controls and from patients with accelerated sedimentation rates having presumably increased fibrinogen levels. An investigation was also made of the practical applicability of these findings.

From the Division of Laboratories, Jewish Sanitarium and Hospital for Chronic Diseases.
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DETERMINATION OF CLOT DENSITY

Nine parts of blood were mixed with 1 part of M/10 solution of sodium citrate. The clot density according to the foregoing definition was determined utilizing the spectrophotometric modification of the one-stage Quick prothrombin method as follows.

One-tenth (0.1) cubic centimeter of plasma and 0.1 c.c. of fresh thromboplastin were placed in a water bath at 37° C. for ten minutes. The tube employed, measuring 6 mm. in diameter and 7.5 cm. in length, was then placed into the microadapter of a Coleman Junior spectrophotometer, Model 6A, with the wave length adjusted at 500 mμ. Then 0.1 c.c. of M/50 solution of $CaCl_2$ was blown swiftly with a 0.1 c.c. blowout pipette into the cuvette which was removed quickly, shaken three times, and replaced in the microadapter. The light beam moved back into the scale and came to a standstill. At this point the optical density was read on the (upper) optical density scale and recorded. At the moment of clotting the light beam suddenly began to move to the left. When it again came to a standstill at the completion of clotting, the final optical density was read and recorded, and the first value subtracted from the second reading. The difference multiplied by 100 represents the clot density.

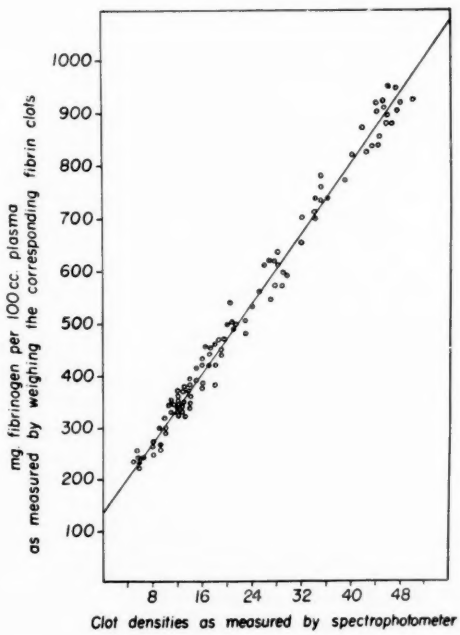

Fig. 1.—Scattergram representing the relationship between the fibrinogen content of plasma and the corresponding clot density in 100 specimens. The straight line can be expressed by the formula

$F = 18\ d + 120$
F = Milligrams fibrinogen in 100 c.c. of plasma
d = Clot density.

IN VITRO STUDIES

(1) Three cubic centimeters of plasma of a series of thirty-six normal individuals and sixty-four acutely or chronically ill patients were clotted with 80 NIH units of topical thrombin (Parke, Davis & Company) and the quantity of fibrinogen was determined gravimetrically.[3] The clot density of each sample as already described was read from the Coleman spectrophotometer and then plotted against the concentration of fibrinogen per 100 c.c. of plasma. The

scattergram thus obtained (Fig. 1) shows that the fibrinogen concentration per 100 c.c. of plasma increases in direct proportion with the clot density. The corresponding points are distributed around a straight line which can be represented by the formula:

$$F = 18\ d + 120$$

F = Milligrams of fibrinogen per 100 c.c. of plasma
d = Clot density

This is in accordance with theoretical conclusions reached later, which establish a linear relationship between the concentration of fibrinogen and the clot density. In order to obtain further proof of this relationship, the following experiments were done.

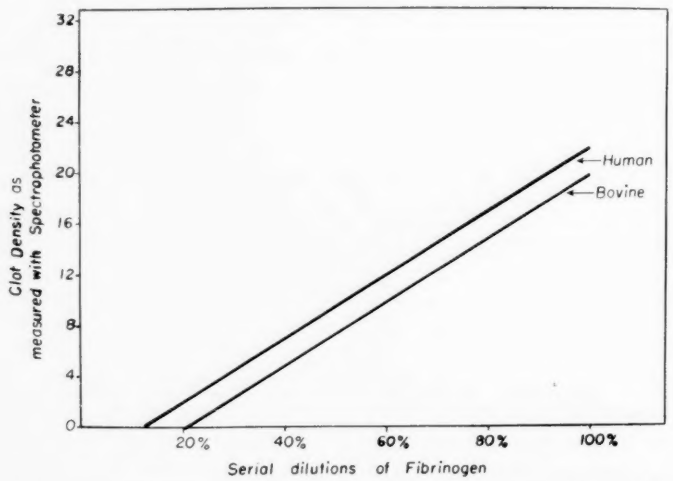

Fig. 2.—Relationship between various concentrations of human and bovine fibrinogen solutions and the corresponding clot densities.

(2) One-tenth (0.1) gram of human fibrinogen and of bovine fibrinogen* was dissolved in 25 c.c. of saline each. These solutions were termed 100 per cent. Subsequent dilutions in saline were made up at 80 per cent, 60 per cent, 40 per cent and 20 per cent concentration of the original solution. Two-tenths (0.2) cubic centimeter of each concentration of human or bovine fibrinogen was then clotted with 20 NIH units of topical thrombin and the clot density was determined. The clot densities were then plotted against fibrinogen concentration as expressed in percentage of the original solution. It was found that the results for both human as well as bovine fibrinogen were similar, inasmuch as the clot density increased quantitatively, and in proportion to the increase of the fibrinogen concentration (Fig. 2).

(3) In a series of eight experiments the quantity of fibrinogen of a given plasma was progressively decreased by diluting it with the defibrinated plasma derived from the same specimen of blood. The fibrinogen was removed from

*We should like to express our thanks to Dr. E. J. Cohn, of Harvard University Medical School, for supplying the human fibrinogen for this study.

5 c.c. of plasma by addition of 100 NIH units of topical thrombin followed by centrifugation and separation of the supernatant defibrinated plasma. This was added to the untreated plasma in various amounts so that concentrations of fibrinogen of 20 per cent, 40 per cent, 60 per cent, 80 per cent, and 100 per cent in plasma were obtained. The clot density according to the described method was then determined for each sample and plotted against the corresponding relative fibrinogen concentration (Fig. 3).

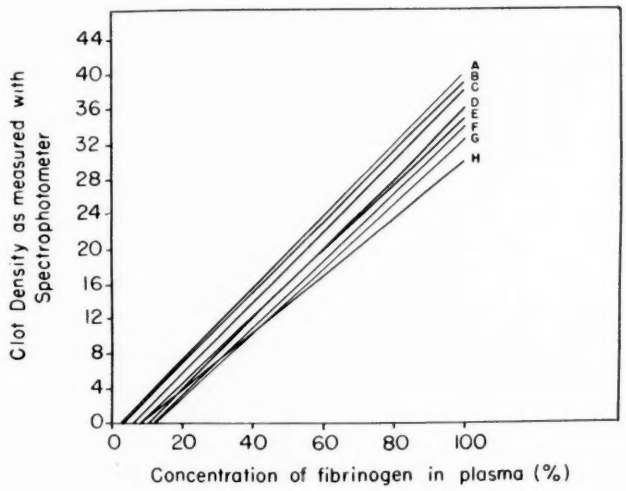

Fig. 3.—Comparison of the fibrinogen concentrations in serial dilutions of human plasma with the corresponding clot density. The initial (100 per cent) fibrinogen concentrations for each specimen of plasma are as follows: *A* = 840 mg. per cent; *B* = 822 mg. per cent; *C* = 804 mg. per cent; *D* = 768 mg. per cent; *E* = 750 mg. per cent; *F* = 732 mg. per cent; *G* = 696 mg. per cent; *H* = 660 mg. per cent.

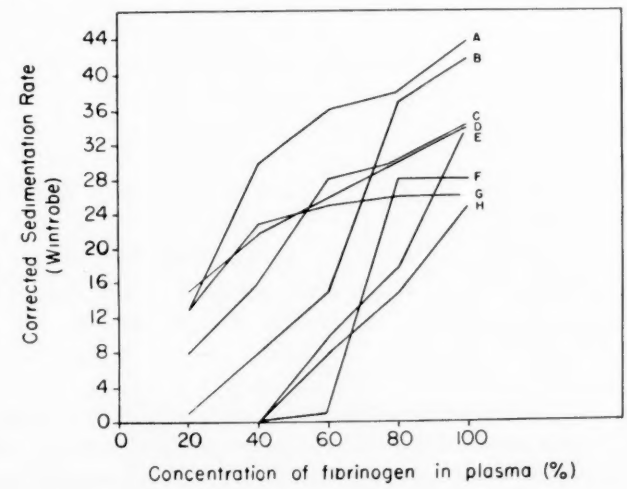

Fig. 4.—Comparison of the fibrinogen concentrations in serial dilutions of human plasma with the corresponding corrected sedimentation rates. The initial (100 per cent) fibrinogen concentrations for each specimen of plasma are the same as in Fig. 3: *A* = 840 mg. per cent; *B* = 822 mg. per cent; *C* = 804 mg. per cent; *D* = 768 mg. per cent; *E* = 750 mg. per cent; *F* = 732 mg. per cent; *G* = 696 mg. per cent; *H* = 660 mg. per cent.

(4) A modification of the preceding experiment was carried out as follows. After preparation of the various dilutions of plasma with homologous defibrinated plasma as already described, the red blood cells from the same specimen of blood were resuspended in the particular samples of plasma containing the various concentrations of fibrinogen. The quantity of the red blood cells, which had been washed three times in saline solution before being resuspended, was determined by the previously established hematocrit reading in Wintrobe tubes. Sedimentation rates were then performed according to the corrected Wintrobe method with each suspension. The resulting sedimentation rates were plotted against the relative fibrinogen concentrations of the corresponding plasma samples (Fig. 4).

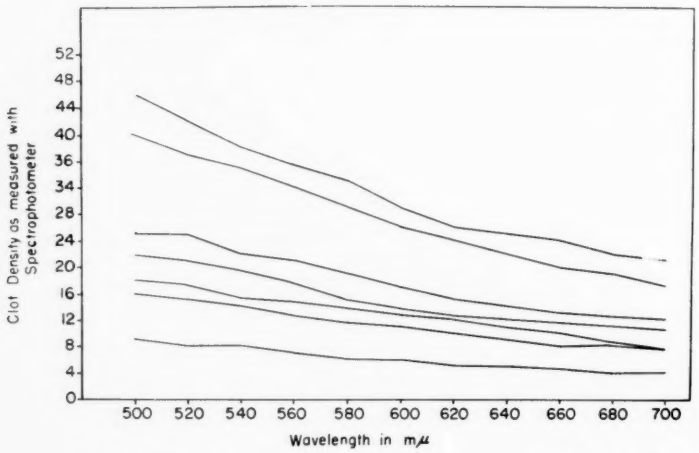

Fig. 5.—Clot densities determined at various wave lengths.

A comparison of Figs. 3 and 4 demonstrates that whereas the clot density varied proportionately with the fibrinogen concentration, the sedimentation rate did not, in general, follow a uniform pattern.

(5) Seven specimens of plasma were tested for clot density at wave lengths varying from 500 mμ to 700 mμ. The obtained clot densities were plotted against the wave lengths (Fig. 5).

The results show that the clot density is lowest at the highest wave length, namely 700 mμ, and highest for the lower wave length of 500 mμ. The latter was selected, therefore, for the determination of the clot density.

CLINICAL STUDY

(1) The corrected sedimentation rate, as well as the clot density, was determined in 450 patients, and the values were plotted against each other (Fig. 6). The mean values of all corrected sedimentation rates for any given clot density were then determined and registered graphically. In general, a close correlation was found between the clot density and the means of the respective corrected sedimentation rates.

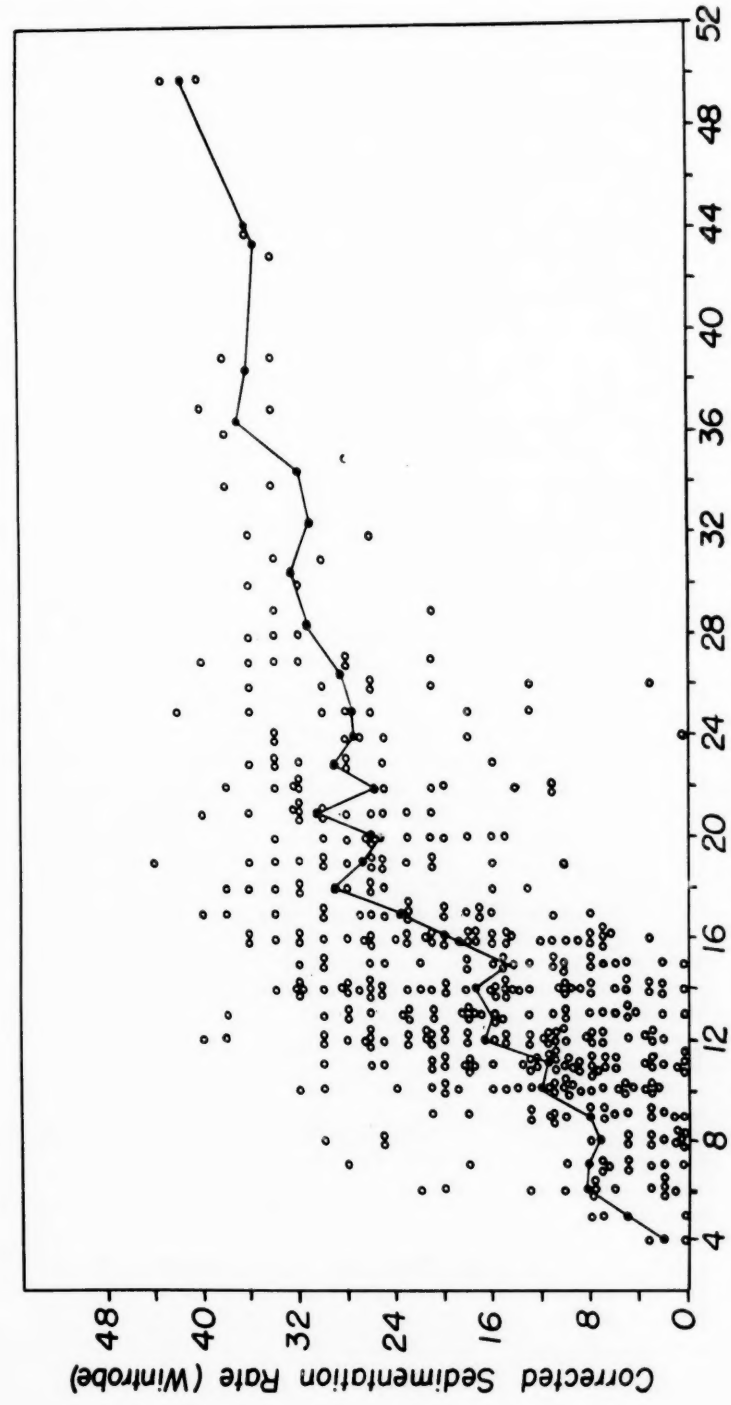

Fig. 6.—Comparison of clot densities and corrected sedimentation rates in a survey of 450 patients.

In fifty-six cases a normal clot density was observed in the presence of an elevated corrected sedimentation rate. On the other hand, where the clot densities were high the corrected sedimentation rates were generally accelerated with the exception of a few points of elevated clot density associated with very low corrected sedimentation rates. These represented two cachectic patients with decubitus ulcers. The fifty-six points indicating a normal clot density combined with an increased corrected sedimentation rate were represented by thirty patients who were on anticoagulant therapy (Dicumarol) and fifteen patients who displayed signs of acute or subacute hepatic involvement. The remaining eleven patients had been hospitalized with various diagnoses such as diabetes mellitus, hypertensive and arteriosclerotic cardiovascular disease.

(2) In a series of twenty acutely ill patients the variations of the corrected sedimentation rate as determined every other day were compared graphically with the corresponding clot densities. With very few exceptions it was found that the clot density followed a pattern similar to that of the corrected sedimentation rate. In computing the mean values a marked parallelism of both tests became evident (Fig. 7).

DISCUSSION

The foregoing studies have shown that a quantitative relationship exists between the fibrinogen concentration in the plasma and the clot density as defined. It was attempted, therefore, to establish a theoretical basis for these findings.

Terming the concentration of all light-absorbing solutes in the plasma before clotting "c_1" and the concentration of all light-absorbing solutes in the plasma after clotting "c_2," the difference then is the concentration of fibrin, c_f.

$$c_2 - c_1 = c_f$$

under the assumption that this process follows the laws of Lambert and Beer.

If T_1 is the transmittancy of the plasma before clotting, then

$$T_1 = \frac{I_1}{I}$$

where I_1 is the light falling onto the photoelectric cell after passage through the plasma before clotting and I the light entering the plasma before clotting.

Similarly, if T_2 is the transmittancy of the plasma after clotting, then

$$T_2 = \frac{I_2}{I}$$

where I_2 is the light falling onto the photoelectric cell after passage through the clotted plasma and I the light entering the clotted plasma.

According to the laws of Lambert and Beer:

$$T_1 = e^{\frac{-c_1}{k}}$$

and

$$T_2 = e^{\frac{-c_2}{k}}$$

Therefore

$$\frac{-c_1}{k} = \ln T_1$$

and

$$\frac{-c_2}{k} = \ln T_2$$

Then

$$\frac{e_2 - e_1}{k} = \ln T_1 - \ln T_2$$

$$e_f = K(\log T_1 - \log T_2)$$

The optical density D_1 of the plasma before clotting is defined as:

$$D_1 = -\log T_1$$

Similarly, the optical density D_2 of the plasma after clotting is defined as:

$$D_2 = -\log T_2$$

Therefore

$$e_f = K\ (D_2 - D_1)$$

The clot density d as defined above is:

$$d = D_2 - D_1$$

Therefore

$$e_f = K \cdot d$$

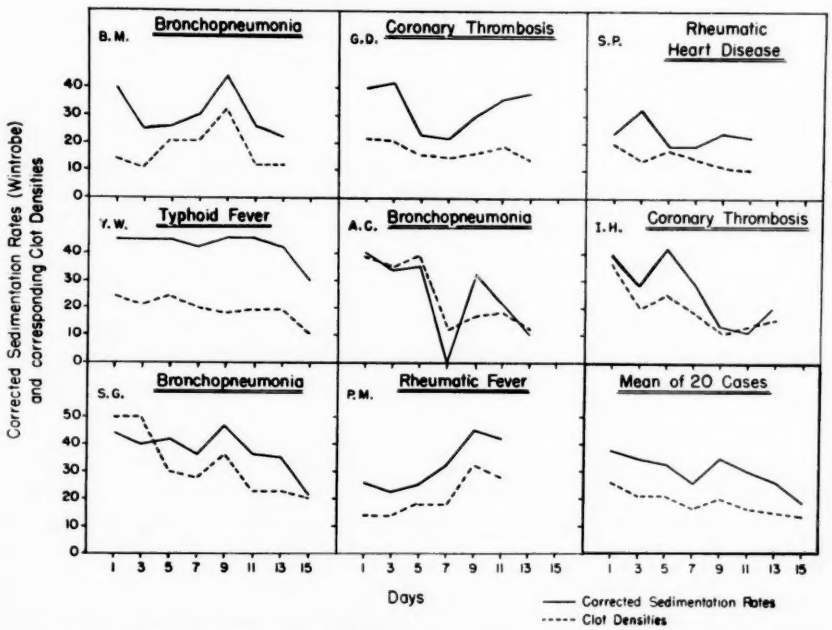

Fig. 7.—Comparison of consecutive changes in the clot density and corrected sedimentation rate in acutely ill patients.

If we assume that a minimal content of fibrinogen is indispensable below which no clot density as a physical phenomenon is obtainable, then the product Kd has to be increased by the expression C which is the fibrinogen concentration at the hypothetical clot density O. The final formula is therefore

$$e_f = K \cdot d + C$$

This formula establishes a linear relationship between the clot density and the amount of fibrin formed in the process of clotting. Since it is assumed that "fibrinogen is the plasma protein which forms fibrin under the action of thrombin,"[4] it is believed that a quantitative identity exists between fibrin and fibrinogen. The foregoing formula, therefore, also gives a relationship between clot density and the fibrinogen content of the plasma.

This theoretical deduction was substantiated by the previously described in vitro experiments. A further verification of the formula was obtained by determining the amount of fibrinogen gravimetrically[3] in 100 specimens of plasma and comparing it with the clot density of the corresponding plasma (Fig. 1).

The formula $F = 18\ d + 120$ yields a normal clot density varying from 5 to 15 which corresponds to a normal fibrinogen concentration of 200 to 400 mg. per 100 c.c. of plasma.[5] A comparison of the clot density between both sexes showed no difference.

The photoelectric determination of fibrinogen has previously been attempted. Thus, Nygaard and co-workers,[6, 7] after registering the process of clotting in an especially constructed apparatus called the photelgraph, determined the fibrinogen content of plasma with the aid of an exponential formula. Another method is that of Nanninga[8] who used turbidimetric measurements after salting out of fibrinogen with ammonium sulfate. It has been shown that the various methods of fibrinogen determination, such as electrophoresis, salting out or clotting, do not necessarily affect the same portion of plasma proteins and that an overlapping of the various fractions exists.[9] Clotting with thrombin, however, seems to come closest to the actual identification of fibrinogen.

In the attempt to compare the clot density with the corrected sedimentation rate, the conclusion reached by Morrison[10] that no direct proportionality exists between the latter and the fibrinogen concentration was confirmed by this study. This can be expected since the sedimentation rate depends upon other factors such as globulin,[11, 12, 13] cholesterol,[14] viscosity,[2, 11] size of red blood cells,[2, 11] or rouleaux formation,[2, 11, 15, 16, 17] besides the concentration of fibrinogen.[2, 18]

A comparison between clot density and corrected sedimentation rate as studied in 450 patients (Fig. 6) shows discrepant values in a series of cases, particularly in patients with acute, subacute, or chronic hepatic disease, and in dicumarolized patients who displayed a normal clot density while the sedimentation rate was increased. Reports of acceleration of the sedimentation rate by Dicumarol administration seems to explain this discrepancy.[19] Hepatic damage, however, can hardly account for it. The scattergram (Fig. 6) also demonstrates a decidedly elevated clot density associated with a normal sedimentation rate in two patients who were markedly cachectic and had large bed sores. These findings are in accord with previous reports that a normal sedimentation rate does not rule out a pathologic process,[20] especially in cachexia.[21]

In comparing the clot density with the corrected sedimentation rate of twenty acutely ill patients over a period of about two weeks, it was found that the course of both was rather similar (Fig. 7).

The utilization of the clot density as a clinical test seems to be of practical value in the case of anticoagulant therapy. It has been shown that in coronary thrombosis the serial changes of the concentration of fibrinogen run the

former parallel to the sedimentation rate[22] and that it also indicates the extent of the myocardial lesion. The clot density, as an expression of the fibrinogen content, can serve, therefore, as a clinical guide in the treatment of coronary thrombosis. From our present material it would appear that the clot density mirrors the pathologic picture more exactly than does the sedimentation rate. A study of this point on a larger number of patients is in progress. Moreover, a practical advantage of this procedure is the fact that the clot density can be read instantaneously, while the prothrombin time is being determined with the spectrophotometer.

SUMMARY

The clot density constitutes the difference of the optical densities of plasma before and after clotting.

It can readily be determined spectrophotometrically utilizing a modification of the one-stage Quick prothrombin method and has been found to be directly proportional to the fibrinogen concentration of the plasma.

A comparison of the clot density with the corrected sedimentation rate was carried out on 450 patients. It was found that a general correlation exists between the two determinations.

A comparison of the clot density with the corrected sedimentation rate in acutely ill patients performed consecutively every other day revealed a closely similar pattern for both tests.

The clot density as a clinical test seems to be of practical value in patients undergoing anticoagulant therapy.

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BIOCHEMICAL STUDIES ON LEUCOCYTES

I. Phosphatase Activity in Health, Leucocytosis, and Myelocytic Leucemia

William N. Valentine, M.D., and William S. Beck, M.D.
Los Angeles, Calif.

INTRODUCTION

IN RECENT years, biochemical and enzymatic activity of animal tissues has aroused increasing interest and investigative study. With the realization that classical morphology has limitations in estimating physiologic activity, increasing attention has been paid to measurable metabolic functions evaluated by cytochemical techniques. The leucocyte is a freely accessible body cell, intimately associated with such commonly encountered circumstances as infectious disease, and is the source of profound metabolic disturbance in such malignant conditions as the leucemias. It has been studied sporadically as a component of whole blood and by techniques aimed at its separation from the other circulating components. Early workers studying the respiratory and energy metabolism, by means of manometric techniques, showed that leucocytes consume O_2,[1] split sugar, and produce lactic acid from glucose.[2] Kempner[3] has reviewed these contributions on differences in aerobic and anaerobic metabolism exhibited by normal and leucemic cells including the earlier work of Soffer and Wintrobe.[4] A variety of biochemical constituents such as ascorbic acid,[5] glutathione,[6] histamine,[7, 8] glycogen,[9-12] and activity indicative of the presence of amylase, lysozyme, lipase, proteolytic ferments, catalase, nucleotidase[13, 14] phosphatase,[15-17] and β-glucuronidase[18, 19] have been found in animal or human leucocytes.

With the advent of improved cytochemical techniques, intracellular localization of lipids, glycogen, and various enzyme centers[20] such as those for phosphatase became possible within certain limitations. In addition, some evidence of quantitative variations of the amount of these substances in health, infection, and leucemia became deducible, and a better concept as to the biochemical constitution of various cell types and cell stages could be formulated. Numerous studies on the histochemistry of blood and bone marrow smears and fixed tissue preparations have appeared in recent years and the availability of several extensive reviews[15, 20, 21] precludes their detailed discussion in this report.

Comparatively few studies, however, have been conducted to measure *quantitatively,* and with controlled clinical and hematologic observations, the innumerable metabolic parameters with which it is now possible to characterize the leucocytes in health, in infection and inflammation of various types, and in the leucemias. This has been partly due to the fairly recent expansion in the num-

From the School of Medicine, University of California at Los Angeles.

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ber of readily performed cytochemical or enzymatic techniques, partly to the preoccupation in the past of many workers in the field of enzymology with other of the body tissues, partly to the frequent separation of adequate clinical material and the biochemical laboratory, and perhaps most of all to the relative difficulty of preparing adequately separated leucocyte material from whole blood until comparatively recently. This latter obstacle has been largely surmounted by the development of a number of separation techniques,[22, 23, 24] the most valuable of which in our hands has been that of increased erythrocyte sedimentation mediated by the addition of fibrinogen or Fraction I solutions of suitable concentration to whole blood.[22] The earlier investigations of oxidative and anaerobic leucocyte metabolism as summarized by Kempner[3] have been mentioned. Wagner[9] has quantitatively estimated the glycogen content of leucocytes in the normal subject and in individuals with leucemia and polycythemia. Little or no glycogen increase was found to occur in chronic lymphatic leucemia and glycogen content was low in acute leucemias. In chronic myelocytic leucemia, there was a considerable increase in glycogen in almost linear proportionality with the increase in the number of cells. However, in polycythemia vera both total and unit cell glycogen were well above normal. Gibson and associates[25] (quoted by Vallee and Altschule[26]) found the zinc content per unit of leucocytes to be sharply reduced in leucemic subjects with elevated counts, and a rise in unit cell zinc content to follow therapeutically induced reduction in the total leucocyte count. Valentine, Pearce, and Lawrence[8] have shown recently that the histamine content of myeloid leucocytes per milliliter of blood is not elevated in polymorphonuclear leucocytosis and may even be lower than normal, while the unit cell histamine is markedly reduced. However, in chronic myelocytic leucemia, histamine values per milliliter of blood as high as 250 times the upper limits of normal have been observed and unit cell histamine is increased on the average by a factor of two and sometimes by more. Furthermore, this metabolic pattern is evident after radiation therapy has restored the total count to the normal range and in cases where the differential leucocyte picture is temporarily indistinguishable from normal. The metabolic abnormality involved, therefore, is very uniform and not dependent merely on cell numbers or immaturity. Rather it appears characteristic of the disease. In polycythemia vera, a pattern tending in the direction of that seen in myelocytic leucemia is evident. Cantopoulos[6] recently has shown quantitative unit cell increases in –SH substances of separated leucocytes in leucemia as compared to the normal state, and, following epinephrine, the –SH content of the white cells of leucemic patients increases to an appreciably greater extent than that of normal persons.

The suggestion that phosphatase may be contained in leucocytes was made by Kay[27] in 1929. Roche[28] succeeded in demonstrating alkaline phosphatase in white blood cell preparations containing both granulocytes and lymphocytes from the rabbit, guinea pig, and horse in 1931. In the same year, Umeno[29] measured phosphatase activity in a suspension of leucocytes obtained as a peritoneal exudate from a rabbit given hypertonic saline solution intraperitoneally, according to the method of de Haan.[30] His report did not mention the pH of the incubation or the cell types present in the suspension. Iwatsuru and co-

workers found marked phosphatase activity in the blood of a patient with chronic myelocytic leucemia[31] and normal values in acute lymphatic leucemia.[32] They also concluded[33] that eosinophils as well as neutrophils contain large amounts of alkaline phosphatase.

Fiessinger and Boyer[34] concluded that the leucocytes are probably not the source of serum phosphatase because they were unable to increase the phosphatase of serum by adding to it a large number of leucocytes taken from the buffy coat of a patient with myelocytic leucemia. They stated that these cells were so poor in phosphatase that undoubtedly their phosphatase content was derived by absorption from the plasma.

While studying tissue sections prepared to show the presence of alkaline phosphatase, Gomori[35] and Fell and Danielli[36] noted evidence of marked activity of this enzyme in the leucocytes. Gomori[37] also found activity in the leucocytes of guinea pig blood smears and, to a lesser degree, in leucocytes of the rat, rabbit, and ground hog. Greenstein[38] and Woodard[39] showed alkaline phosphatase to be present in bone marrow by histochemical techniques. Kabat and Furth[40] reported contradictory results, finding weakly positive alkaline phosphatase activity only in the endothelial cells of bone marrow. Wachstein,[17] studying smears of human blood, found varying proportions of alkaline phosphatase positive neutrophilic leucocytes. The intensity of reaction increased markedly in infection. Alkaline phosphatase activity was clearly present in large amounts in bone marrow suggesting that phosphatase is already present in the leucocytes before they are released from the marrow. In patients with chronic myelocytic leucemia, phosphatase activity was depressed in the neutrophils.

The work of Rossiter[41] demonstrated that surface-active substances such as saponin, bile salts, and alkyl sulfate detergents liberate large amounts of active phosphatase from leucocytes in suspension. Employing saponin as the surface-active agent, Cram and Rossiter[42] carefully studied the phosphatase of rabbit polymorphonuclear leucocytes obtained from peritoneal exudate by the method of de Haan.[30] The pH activity curve disclosed 2 maxima, one in the region of pH 10 and one at pH 5.0. The course of the hydrolytic reaction was found to be linear in the first sixty minutes. Mg^{++} and glycine in low concentrations were observed to activate the phosphatase, and inhibition was caused by Zn^{++}, CN^-, borate, phosphate, bile salts, and glycine in high concentrations. Haight and Rossiter[16] obtained human leucocytes by the constricted tube method of Butler and Cushman[5] and by flotation in gum acacia. Using disodium phenylphosphate as the substrate, they found alkaline phosphatase to be confined chiefly to the polymorphonuclear leucocytes and acid phosphatase chiefly to the lymphocytes, although present also in lesser concentration in the polymorphonuclear leucocytes. They noted a quantitative difference between the leucocytes of the rabbit and man, the former having as much as eight times the alkaline phosphatase activity and one-half the acid phosphatase activity per cell as the latter. These authors restricted their observations to normal human leucocytes, recording no data on leucocytotic or leucemic states.

The emergence of varying metabolic patterns of leucocytes with different types of disease processes makes it imperative to define as many as possible of

the parameters which characterize each set of circumstances. It is the purpose of the present report (a) to present data on the quantitative alkaline and acid phosphatase activity of separated leucocytes in normal subjects, in subjects with infection and various inflammatory conditions, and in subjects with chronic myelocytic leucemia and (b) to emphasize the emerging, widely varying metabolic patterns which characterize the leucocytes in various pathologic states. Data on phosphatase activity in lymphatic and stem cell leucemias will be the subject of another report.

MATERIALS AND METHODS

The preliminary separation of leucocytes was accomplished by mixing 20 ml. of venous blood with a freshly prepared saline solution of bovine fibrinogen (Fraction I), Armour, of such concentration that the packed red cell volume of the final solution was approximately 30 volumes per cent, and its fibrinogen content 7 mg. per milliliter.[22] The citrate content of the original fibrinogen preparation was high enough to constitute an adequate anticoagulant. All foam was carefully removed from the mixture before allowing it to stand. After twenty to thirty minutes most of the red cells had sedimented, permitting the removal of a supernatant plasma suspension of leucocytes and platelets which was largely free of erythrocytes. The supernate was centrifuged for three minutes at 800 r.p.m., the plasma removed, and the cells resuspended in saline. Centrifugation and washing were repeated and the final concentration of cells in the suspension was determined by standard leucocyte counts done in quadruplicate. Platelet contamination was eliminated to a large extent by the differential centrifugation.

Incubation mixtures for the determination of phosphatase activity consisted of the following:

ALKALINE PHOSPHATASE DETERMINATION	AMOUNT	FINAL CONC. IN INCUBATION MIXTURE
Sodium β-glycerophosphate solution* (0.026M, buffered with NaOH and sodium diethyl barbiturate to pH 9.9)	9.0 ml.	0.02M
Saponin (2% in saline)†	0.5 ml.	100 mg. %
$MgCl_2$ (0.050M)	0.2 ml.	0.001M
Leucocyte suspension	0.3 ml.	
Total volume	10.0 ml.	

ACID PHOSPHATASE DETERMINATION	AMOUNT	FINAL CONC. IN INCUBATION MIXTURE
Sodium β-glycerophosphate solution (0.026M, buffered with acetic acid and sodium diethyl barbiturate to pH 5.0)	9.0 ml.	0.02M
Saponin (2% in saline)	0.5 ml.	100 mg. %
$MgCl_2$ (0.050M)	0.2 ml.	0.001M
Leucocyte suspension	0.3 ml.	
Total volume	10.0 ml.	

*Eastman Organic Chemicals.
†British Drug Houses, Ltd.

All determinations were run in duplicate. The reactions were terminated after sixty minutes' incubation at 37° C. by the addition of 2 ml. of 30 per cent trichloroacetic acid. Precipitated material was removed by filtration. Sample blanks consisted of mixtures to which the trichloroacetic acid was added prior to the aliquot of cells, and reagent blanks contained 0.3 ml. of saline solution in place of the cell suspension. Phosphorus was determined in the filtrates by the method of Fiske and SubbaRow.[13] To 3 ml. aliquots of filtrate were added 0.5 ml. of molybdate reagent, 0.5 ml. of amino-naphthol-sulfonic acid reagent, and 4.0 ml. of water. The blue color was measured in a Beckman Model B spectrophotometer at 660 μ.

At the time blood was obtained by venepuncture, total leucocyte counts were also made on capillary blood. Counts were made in duplicate from each of two pipettes and the results averaged. Smears were likewise prepared at this time and differential counts based on 200 cells were subsequently made. The total and differential leucocyte counts permitted calculation of phosphatase activity per milliliter of blood and some assessment of the relative contribution of various cell types to that activity.

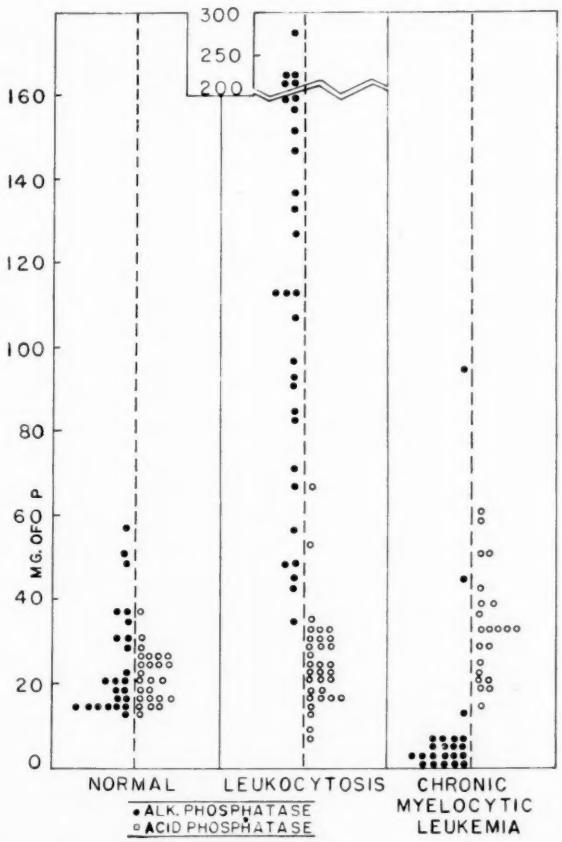

Fig. 1.—Phosphatase activity of leucocytes in normal individuals and subjects with leucocytosis and chronic myelocytic leukemia. Phosphatase activity is here expressed in terms of milligrams of phosphorus liberated per hour by 10^{10} leucocytes in saline suspension. Black dots refer to alkaline phosphatase; circles indicate acid phosphatase. In several instances, more than one determination on a single patient has been recorded. These usually have represented different phases of the disease as indicated in Tables II and III.

PRESENTATION OF RESULTS

General.—Several modes of expression have been used to depict the data. Phosphatase activity, given in terms of milligrams of phosphorus liberated per hour by 10^{10} leucocytes, is a useful expression, despite the fact that the various cell types making up this population may contribute unequally to its total activity. It has been found that the leucocytes in health, infection, and in myelocytic leucemia have characteristic patterns which are most clearly indicated in terms of phosphatase activity per 10^{10} leucocytes. Phosphatase activity has

TABLE I. PHOSPHATASE ACTIVITY IN NORMAL SUBJECTS

SUBJECT	LEUCOCYTE COUNT PER CU.MM. (CAPILLARY BLOOD)	DIFFERENTIAL COUNT			LEUCOCYTE COUNT PER CU.MM. (SUSPENSION)	ALKALINE PHOSPHATASE PER 10^{10} LEUCOCYTES	ALKALINE PHOSPHATASE PER LEUCOCYTES OF 1 ML. OF BLOOD	ACID PHOSPHATASE PER 10^{10} LEUCOCYTES	ACID PHOSPHATASE PER LEUCOCYTES OF 1 ML. OF BLOOD
		GRANULOCYTES	LYMPHOCYTES	MONOCYTES		MG. PHOSPHORUS LIBERATED PER HR.			
W. B.									
8/23/50	6,975	5,475 (78.5)	1,325 (19.0)	174 (2.5)	19,650	35.1	0.025	17.9	0.013
9/29/50	7,163	5,444 (76.0)	1,576 (22.0)	143 (2.0)	16,075	28.0	0.020	18.4	0.013
1/22/51	6,500	4,810 (74.0)	1,430 (22.0)	426 (4.0)	12,988	28.3	0.018	23.6	0.015
L. P.	6,425	4,433 (69.0)	1,735 (27.0)	257 (4.0)	5,725	16.3	0.011	22.3	0.014
J. La.	6,700	5,075 (76.0)	1,407 (21.0)	201 (3.0)	5,125	37.4	0.025	20.2	0.014
R. D.	7,775	5,015 (64.5)	2,488 (32.0)	272 (3.5)	12,400	15.2	0.012	14.2	0.011
C. K.	8,000	4,400 (55.0)	3,320 (41.5)	280 (3.5)	12,188	15.4	0.012	13.7	0.011
E. R.	5,550	4,024 (72.5)	1,388 (25.0)	139 (2.5)	15,000	58.0	0.032	26.3	0.015
D. M.	7,583	4,057 (53.5)	3,412 (45.0)	114 (1.5)	7,625	23.0	0.017	20.9	0.016
K. K.	7,550	4,304 (57.0)	3,171 (42.0)	76 (1.0)	16,075	17.1	0.013	21.8	0.017
C. S.	6,500	4,811 (74.0)	1,593 (24.5)	98 (1.5)	9,275	21.9	0.014	14.2	0.009
M. H.	6,750	4,995 (74.0)	1,552 (23.0)	203 (3.0)	21,663	49.7	0.034	14.4	0.010
W. M.	9,438	6,323 (67.0)	2,595 (27.5)	519 (5.5)	20,463	19.1	0.018	36.8	0.035
J. Le.	5,125	3,588 (70.0)	1,256 (24.5)	282 (5.5)	14,088	15.3	0.008	27.2	0.014
I. W.	5,438	3,480 (64.0)	1,495 (27.5)	462 (8.5)	7,975	28.5	0.016	26.5	0.014
A. L.	9,275	5,287 (57.0)	3,246 (35.0)	742 (8.0)	17,963	16.7	0.015	18.7	0.017
L. B.	8,975	5,475 (61.0)	3,060 (34.0)	450 (5.0)	18,063	20.8	0.019	17.5	0.016
B. L.	5,088	3,104 (61.0)	1,781 (35.0)	204 (4.0)	12,675	15.1	0.008	24.3	0.012
J. H.	6,463	3,490 (54.0)	2,585 (40.0)	388 (6.0)	9,363	19.6	0.013	17.1	0.011
R. P.	5,013	2,557 (51.0)	2,005 (40.0)	451 (9.0)	14,025	13.4	0.007	28.7	0.014
J. N.	8,438	4,852 (57.5)	3,040 (36.0)	548 (6.5)	16,933	32.5	0.027	16.5	0.014
J. T.	6,088	4,180 (68.0)	1,523 (25.0)	427 (7.0)	12,313	31.4	0.019	31.4	0.019
J. F.	6,488	3,568 (55.0)	2,516 (39.0)	388 (6.0)	9,563	21.7	0.014	24.6	0.016
B. G.	6,800	4,060 (60.0)	2,444 (36.0)	672 (4.0)	10,688	50.8	0.035	26.5	0.018
E. J.	5,200	4,160 (80.0)	884 (17.0)	156 (3.0)	9,113	15.8	0.008	25.0	0.013
Mean						25.8	0.018	21.9	0.015

also been referred to the leucocytes of 1 ml. of blood. The data lend themselves to concise graphical and tabular presentation. Fig. 1 indicates, on a scattergram, all alkaline and acid phosphatase values in the three groups of cases. Tables I, II, and III present the data in numerical form with information on counts and differentials.

Normals.—In a group of twenty-three normal individuals, the alkaline phosphatase activity expressed as previously indicated per 10^{10} cells ranged from 13.4 to 58.0. The mean level was 25.8 and the median 19.4. Repeated determinations in a single individual (W. B.) showed the activity to be quite constant. The acid phosphatase measurements per 10^{10} leucocytes varied from 13.7 to 36.8. The mean acid phosphatase was 21.9 and the median 21.8. The range of activity in normal subjects was distinctly narrower in the acid than in the alkaline enzyme.

The alkaline phosphatase activity in terms of milligrams of phosphorus liberated per hour by the leucocytes of 1 ml. of blood was found to be 0.007 to 0.035, the mean being 0.018. The corresponding values for acid phosphatase ranged from 0.009 to 0.035, with a mean of 0.015.

Leucocytosis.—This group consisted of thirty persons with white blood cell counts ranging from 6,488 to 32,750. Those with levels below 11,988 (K. B., D. Sa., and N. S.) are patients whose leucocyte counts had been greatly elevated within the previous twenty-four to forty-eight hours. It will be noted that these patients, despite a normal total and differential leucocyte count, still maintained abnormal levels of phosphatase activity. The etiology of the leucocytoses varied, the larger number having some type of infection. The remainder had miscellaneous conditions such as myocardial infarction, hemorrhage, or trauma.

Fig. 1 and Table II show the greatly elevated levels of alkaline phosphatase in the patients with leucocytosis, the range extending from 35.4 to 276.5 with a mean of 119.1, nearly five times that of normal subjects. It is interesting to note that in the patients whose leucocytosis was associated with no clinically obvious infection, a group of six individuals, alkaline phosphatase levels varied from 42.9 to 96.7 with a mean of 66.4. Conversely, the activity levels of patients who had obvious infections ranged from 35.4 to 276.5 with a mean of 132.3. It can be seen that of the group whose leucocyte counts had only recently returned to normal from leucocytotic levels, all had markedly elevated alkaline phosphatase activity. It may also be mentioned that two persons (S. S. and O. M.) had severe and extensive infections and were, in fact, terminal. These two had alkaline phosphatase levels in the lower ranges (66.7 and 57.6) of the group with infections.

The acid phosphatase per 10^{10} cells in subjects with leucocytosis tended to remain approximately within the normal range, varying from 6.6 to 66.4 with a mean of 25.7. In spite of the greatly increased percentage of myeloid cells and the corresponding relative diminution of lymphoid cells in leucocytoses of the type studied, these observations indicate no substantial alteration in unit cell activity from that observed in normal subjects. This might be construed as meaning that acid phosphatase activity is distributed in some roughly equal manner between the various leucocyte types so that wide shifts in the differential

TABLE II. PHOSPHATASE ACTIVITY IN INDIVIDUALS WITH LEUCOCYTOSIS (THOSE MARKED WITH AN ASTERISK ARE PATIENTS WHO SHOWED NO CLINICAL EVIDENCE OF INFECTION)

SUBJECT	LEUCOCYTE COUNT PER CU.MM. (CAPILLARY BLOOD)	DIFFERENTIAL COUNT			LEUCOCYTE COUNT PER CU.MM. (SUSPENSION)	MG. PHOSPHORUS LIBERATED PER HR.				CLINICAL STATUS
		GRANULOCYTES	LYMPHOCYTES	MONOCYTES		ALKALINE PHOSPHATASE PER 10^{10} LEUCOCYTES	ALKALINE PHOSPHATASE PER LEUCOCYTES OF 1 ML. OF BLOOD	ACID PHOSPHATASE PER 10^{10} LEUCOCYTES	ACID PHOSPHATASE PER LEUCOCYTES OF 1 ML. OF BLOOD	
K. B.										
10/20/50	15,663	12,530 (80.0)	2,428 (15.5)	705 (4.5)	31,600	138.2	0.217	16.4	0.026	Acute pharyngitis
10/23/50	6,488	3,698 (57.0)	2,465 (38.0)	324 (5.0)	16,275	226.9	0.147	66.4	0.043	Successfully treated with penicillin
D. Sa.	7,525	4,929 (65.5)	2,258 (30.0)	339 (4.5)	8,863	132.7	0.100	17.6	0.013	Pneumonitis: W.B.C. 24,400 48 hr. previously
N. S.	7,650	6,120 (80.0)	1,377 (18.0)	153 (2.0)	26,438	163.4	0.125	54.5	0.042	Pneumonitis: W.B.C. 25,500 24 hr. previously
S. S.	25,167	22,524 (89.5)	2,643 (10.5)	0 (0)	47,550	66.8	0.168	33.6	0.084	Meningitis, terminal
O. M.	19,813	18,228 (92.5)	1,090 (5.5)	396 (2.0)	33,775	57.6	0.114	16.8	0.033	Meningitis, terminal
O. F.	15,450	12,051 (78.0)	3,090 (20.0)	309 (2.0)	44,714	113.9	0.176	21.6	00.33	Acute pyelonephritis
P. B.	16,583	15,432 (81.0)	2,985 (18.0)	166 (1.0)	21,016	159.2	0.264	33.4	0.055	Pneumonitis, uncontrolled diabetes
J. D.	16,633	14,304 (86.0)	1,996 (12.0)	333 (2.0)	24,275	221.3	0.368	32.4	0.054	Acute bronchopneumonia
J. G.	20,238	17,809 (88.0)	1,821 (9.0)	607 (3.0)	47,850	115.4	0.233	19.9	0.040	Infected surgical incision
C. J.	18,933	16,093 (85.0)	2,461 (13.0)	379 (2.0)	26,600	92.2	0.175	6.6	0.013	Cystitis, skin infection, diabetes
T. O.	13,013	10,475 (80.5)	1,887 (14.5)	651 (5.0)	30,600	90.8	0.118	14.0	0.018	Pneumonitis
W. D.	14,288	12,992 (91.0)	1,286 (9.0)	0 (0)	16,225	276.5	0.395	8.4	0.012	Pneumonitis
C. F.	32,750	29,966 (91.5)	2,784 (8.5)	0 (0)	33,075	147.1	0.482	16.0	0.053	Infected surgical incision

G. S.	16,138	12,910 (80.0)	1,775 (11.0)	1,452 (9.0)	16,763	193.2	0.312	25.2	0.041	Pneumonitis, empyema
R. M.	25,125	23,366 (93.0)	1,759 (7.0)	0 (0)	48,750	70.0	0.176	22.2	0.056	Urinary infection, cerebral thrombosis
J. E.	16,763	13,578 (81.0)	2,012 (12.0)	1,173 (7.0)	28,850	48.3	0.081	22.0	0.037	Acute appendicitis
L. L.	14,975	12,729 (85.0)	1,947 (13.0)	300 (2.0)	23,988	113.4	0.170	30.4	0.047	Acute appendicitis
A. L.	11,988	8,272 (69.0)	3,596 (30.0)	120 (1.0)	25,450	35.4	0.043	20.1	0.024	Postop. appendectomy
J. W.	15,125	14,218 (94.0)	908 (6.0)	0 (0)	33,700	158.5	0.240	32.3	0.049	Hodgkin's disease, fever
J. Wa.	26,638	23,708 (89.0)	2,664 (10.0)	266 (1.0)	35,925	167.3	0.446	28.4	0.076	Acute cholecystitis
P. R.	24,338	23,608 (97.0)	730 (3.0)	0 (0)	49,250	127.0	0.309	16.2	0.039	Pneumonitis
J. R.	22,200	19,647 (88.5)	2,331 (10.5)	111 (0.5)	36,975	107.3	0.238	27.5	0.061	Diabetes, bronchopneumonia
X. A.	18,625	15,625 (84.0)	932 (5.0)	2,050 (11.0)	29,300	152.0	0.283	25.5	0.047	Pneumonitis
G. H.*	14,163	11,685 (82.5)	2,337 (16.5)	142 (1.0)	26,763	42.9	0.061	32.8	0.046	Hematemesis treated with I.V. fluids
J. Da.*	13,950	11,788 (84.5)	2,162 (15.5)	0 (0)	49,875	84.4	0.118	30.9	0.043	Multiple fractures
A. R.*	17,900	15,215 (85.0)	2,327 (13.0)	358 (2.0)	64,500	44.4	0.080	18.7	0.034	Carcinoma of tongue
A. F.*	17,700	16,550 (93.5)	796 (4.5)	354 (2.0)	42,513	81.3	0.144	21.4	0.038	Postop. exploratory laparotomy
B. S.*	20,450	18,075 (88.0)	2,259 (11.0)	205 (1.0)	47,675	48.8	0.100	35.8	0.074	Myocardial infarction
E. T.*	20,000	17,400 (87.0)	2,600 (13.0)	0 (0)	46,000	96.7	0.193	25.1	0.050	Pulmonary infarction
Mean						119.1	0.203	25.7	0.043	

TABLE III. PHOSPHATASE ACTIVITY IN INDIVIDUALS WITH CHRONIC MYELOCYTIC LEUCEMIA

(Patient Y. A. is presented here although the diagnosis of leucemia is not established. This case and Patient W. H. are excluded from the mean and are referred to in the discussion)

SUBJECT AND DATE	LEUCOCYTE COUNT PER CU.MM. (CAPILLARY BLOOD)	DIFFERENTIAL COUNT						LEUCOCYTE COUNT PER CU.MM. (SUSPENSION)	MG. PHOSPHORUS LIBERATED PER HOUR				REMARKS
		BLASTS	MYELOCYTES	METAMYELOCYTES	STABS	MATURE GRANULOCYTES	LYMPHOCYTES		ALKALINE PHOSPHATASE PER 10^{10} LEUCOCYTES	ALKALINE PHOSPHATASE PER LEUCOCYTES OF 1 ML. OF BLOOD	ACID PHOSPHATASE PER 10^{10} LEUCOCYTES	ACID PHOSPHATASE PER LEUCOCYTES OF 1 ML. OF BLOOD	
W. T.													
9/21/50	3,500	0 (0)	0 (0)	105 (3.0)	105 (3.0)	2,590 (74.0)	665 (19.0)	6,625	7.2	0.003	32.5	0.011	Treated with P^{32}
10/26/50	8,075	2,907 (36.0)	1,696 (21.0)	162 (2.0)	646 (8.0)	1,857 (23.0)	808 (10.0)	28,238	0.0	0.000	28.8	0.023	
1/18/51	16,513	0 (0)	6,275 (38.0)	2,972 (18.0)	1,816 (11.0)	4,789 (29.0)	330 (2.0)	22,625	2.5	0.004	33.8	0.056	
J. P.													
9/25/50	47,733	239 (0.5)	2,148 (4.5)	11,933 (25.0)	12,172 (25.5)	20,525 (43.0)	477 (1.0)	78,500	3.8	0.018	36.0	0.172	Receiving radiation
10/11/50	11,588	0 (0)	695 (6.0)	1,101 (9.5)	985 (8.5)	7,996 (69.0)	695 (6.0)	34,200	4.1	0.005	21.9	0.025	Treatment completed
J. A.													
9/28/50	101,200	0 (0)	12,650 (12.5)	29,854 (29.5)	18,722 (18.5)	36,938 (36.5)	3,036 (3.0)	146,333	1.2	0.012	34.3	0.347	Preradiation
10/12/50	8,488	0 (0)	127 (1.5)	127 (1.5)	1,443 (17.0)	6,366 (75.0)	424 (5.0)	20,075	2.4	0.002	19.9	0.017	Postradiation
11/29/50	94,450	0 (0)	4,250 (4.5)	17,001 (18.0)	22,668 (24.0)	50,059 (53.0)	472 (0.5)	100,583	14.4	0.136	39.2	0.370	Severe pyoderma
12/ 8/50	126,500	633 (0.5)	25,300 (20.0)	24,035 (19.0)	22,138 (17.5)	53,763 (42.5)	633 (0.5)	83,375	6.8	0.086	51.6	0.653	Pyoderma improved
O'N.													
9/21/50	30,138	9,644 (32.0)	3,466 (11.5)	753 (2.5)	3,767 (12.5)	9,041 (30.0)	603 (2.0)	33,825	4.4	0.013	51.9	0.156	Receiving radiation

11/ 1/50	8,463	169 (2.0)	1,777 (21.0)	1,439 (17.0)	762 (9.0)	3,301 (39.0)	1,016 (12.0)	23,000	4.2	0.004	59.5	0.050	Treatment completed
W. H.													
11/15/50	39,813	0 (0)	0 (0)	0 (0)	1,194 (3.0)	37,822 (95.0)	796 (2.0)	61,250	45.8	0.183	21.1	0.084	
12/13/50	31,217	0 (0)	0 (0)	937 (3.0)	1,249 (4.0)	27,470 (88.0)	1,560 (5.0)	44,800	94.9	0.296	26.5	0.083	
F. B.	88,000	4,840 (5.5)	12,760 (14.5)	13,200 (15.0)	24,640 (28.0)	31,240 (35.5)	880 (1.0)	109,250	4.5	0.039	29.1	0.256	
E. L.	15,088	0 (0)	604 (4.0)	3,319 (22.0)	4,074 (27.0)	6,790 (45.0)	301 (2.0)	19,875	0.0	0.000	32.5	0.049	
T.W.	77,075	0 (0)	6,551 (8.5)	25,820 (33.5)	16,957 (22.0)	26,206 (34.0)	1,542 (2.0)	67,417	2.4	0.018	22.6	0.174	
T. A.	46,438	0 (0)	3,947 (8.5)	9,055 (19.5)	11,377 (24.5)	19,504 (42.0)	1,857 (4.0)	56,750	2.2	0.010	32.5	0.151	
B. S.	165,200	5,782 (3.5)	8,260 (5.0)	59,472 (36.0)	28,910 (17.5)	49,560 (30.0)	13,216 (8.0)	188,620	3.1	0.051	19.1	0.316	
D. S.	15,500	0 (0)	0 (0)	0 (0)	0 (0)	9,920 (64.0)	4,495 (29.0)	27,588	6.9	0.011	15.6	0.024	
M. H.	403,000	4,030 (1.0)	104,780 (26.0)	40,300 (10.0)	112,840 (28.0)	124,930 (31.0)	8,060 (2.0)	183,750	1.1	0.045	38.3	1.542	
W. Ha.	107,000	1,070 (1.0)	35,310 (33.0)	13,910 (13.0)	20,330 (19.0)	34,240 (32.0)	2,140 (2.0)	41,000	6.0	0.065	61.4	0.657	
J. R.	26,800	0 (0)	2,940 (11.0)	3,216 (12.0)	4,556 (17.0)	16,080 (60.0)	0 (0)	24,875	1.6	0.004	43.6	0.117	
Mean (excluding Patient W. H.)									4.0	0.027	35.3	0.269	
(Y. A.)													
10/27/50	13,100	0 (0)	0 (0)	0 (0)	86 (0.67)	8,423 (64.3)	4,585 (35.0)	27,100	3.2	0.004	21.5	0.028	Undiagnosed elevation of leucocyte count (See discussion)
11/ 8/50	16,000	0 (0)	0 (0)	0 (0)	0 (0)	10,720 (67.0)	5,280 (33.0)	27,638	2.6	0.004	17.8	0.028	

formula show no reflection in shifts in the acid phosphatase values per 10^{10} cells. The acid phosphatase activity of leucocytes per milliliter of blood would then be increased in rough proportion to the increase in total leucocytes irrespective of the differential picture. On the other hand, in view of the observations of Haight and Rossiter,[16] it might be argued that acid phosphatase activity resides very largely in the lymphocyte, but that in conditions accompanied by neutrophilic leucocytosis, the absolute reduction in lymphocytes per 10^{10} cells is compensated for by a corresponding increase in acid phosphatase activity per lymphocyte. No difference is noted in regard to acid phosphatase between the groups with "infectious" and "noninfectious" leucocytoses.

On the other hand, the close correlation of elevations of alkaline phosphatase activity with neutrophilic leucocytoses strongly suggests that it resides largely in cells of the granulocytic series. The degree of elevation in activity also gives quantitative substantiation to histochemical evidence of increased unit cell alkaline phosphatase in infection as inferred from properly prepared blood smears.[17] The alkaline phosphatase activity per leucocytes of 1 ml. of blood is extremely high in leucocytosis as a consequence of both the increased leucocyte count and the increased amount of phosphatase per leucocyte. There is also an increase in acid phosphatase expressed in this manner but here the increase is no more than can be accounted for by the increase in leucocyte numbers. As will be shown below, there is further evidence that acid phosphatase does occur in the cells of the myeloid series, and it appears highly likely that the increased acid phosphatase per leucocytes of 1 ml. of blood in neutrophilic leucocytoses is at least partially accounted for by the contributions of cells of the granulocytic series.

Chronic Myelocytic Leucemia.—Twenty-two phosphatase determinations were done on the fourteen patients who made up this group. In the instances where repeated determinations were carried out on the same patient, there had occurred some significant change in the patient's clinical status such as supervening infection, change in white blood cell count, or some form of therapy. This statement points up the difficulty in accumulating a homogeneous group of cases with similar and strictly comparable clinical pictures. It is all the more striking, therefore, that despite the diversity in the clinical and hematologic status of this group, the alkaline phosphatase activities recorded are, with one exception, all far below the lowest values obtained in normal subjects.

The range of alkaline phosphatase activity in this group (excepting Patient W. H.) was 0 to 14.4, the mean being 4.0. There was only one result higher than 7.2 and this was in Patient J. A. who at the time was suffering from an extensive skin infection. Phosphatase levels in this patient before and after infection were considerably lower. It may be interjected that although a rise in leucocyte alkaline phosphatase accompanied severe infection in this patient, the level attained barely reached the lowest level found in normals and was of an entirely different magnitude from the activities accompanying ordinary leucocytosis of infection.

There appeared to be little correlation between the white blood cell count and the alkaline phosphatase activity per 10^{10} cells as is shown in Patients

J. P., J. A., and O'N. Also, it is apparent that the low level of phosphatase activity per 10^{10} cells is not merely a matter of cell immaturity. Two instances are recorded (W. T. and D. S.) in which the patient had a normal complement of mature myeloid cells but had nevertheless a low unit cell alkaline phosphatase, although admittedly these figures (7.2 and 6.9) were not as low as those of other patients with a greater shift toward immaturity in the differential formula. The argument that the lower alkaline phosphatase level per cell is not solely attributable to immaturity is further supported in the expressions of activity per leucocytes of 1 ml. of blood. In many instances (J. P., J. A., F. B., and E. L.) there is a greatly increased number of morphologically mature myeloid cells per milliliter of blood, and yet there is no corresponding increase in the alkaline phosphatase per milliliter of blood. On the contrary, these figures tend to be low and show only moderate increases above normal in the presence of extremely high leucocyte counts (J. A. on 12/8, B. S., and M. H.).

The one great exception to the trend of this group is the data of Patient W. H. This patient, a 67-year-old man, received a diagnosis of chronic myelocytic leucemia in 1948. The diagnosis rested upon the findings of elevated leucocyte count with some shift to the left in the myeloid series, marked splenomegaly, hepatomegaly, and myeloid hyperplasia of bone marrow. Radiation to the spleen resulted in reduction in splenic size and in the leucocyte count. The patient had also received urethane. Throughout his clinical course the leucocyte count has always remained below 60,000 per cubic millimeter and always the shift toward immaturity of the granulocytes has been less than that commonly encountered in myelocytic leucemia. Repeated histamine determinations have indicated values only slightly above normal and are in contrast to all other cases of myelocytic leucemia studied by the histamine assay technique in this laboratory. The marked differences in alkaline phosphatase activity exhibited by the leucocytes of this patient in contrast to that of other patients with similar clinical pictures will be discussed below.

The acid phosphatase activity per 10^{10} leucemic cells can be seen in Fig. 1 to show a tendency toward higher than normal levels, although broadly overlapping the normal group. These figures range from 19.1 to 61.4 with a mean of 35.3, a level distinctly higher than the normal mean. Consequently, the acid phosphatase per cells of 1 ml. of blood is about normal in patients whose leucocyte counts are in the normal range and extremely high in those with high counts. The relationship between count and acid phosphatase activity per cells of 1 ml. of blood tends to be linear because of the narrow range in unit cell acid phosphatase activity values.

In two instances (J. P. and J. A.) there is a suggestion of decreased acid phosphatase levels per 10^{10} cells during periods when leucocyte counts were relatively low. In both of these instances, the number of lymphocytes present in each aliquot of 10^{10} cells was greater at the lower level of leucocyte count. Thus, when J. P. had a white count of 47,733, he had 1.0 per cent lymphocytes; when his count was 11,588, he had 7.0 per cent lymphocytes. J. A. had 0.5 per cent lymphocytes when his count was 94,450 and 5.0 per cent lymphocytes when his count was 8,488. The finding of normal to high acid phosphatase values per

10^{10} cells in the presence of myelocytic leucemia plus the tendency to even a slightly lower unit cell acid phosphatase in these subjects when the lymphocytes were relatively increased, again is suggestive evidence of substantial acid phosphatase activity in myeloid cells.

Thus, there has been described a characteristic pattern in the alkaline and acid phosphatase activity in the leucocytes of chronic myelocytic leucemia and neutrophilic leucocytosis. The cells of the former show low alkaline phosphatase and high normal to high acid phosphatase levels. The latter in striking contrast show extremely high levels of alkaline phosphatase particularly in infection and approximately normal acid phosphatase levels. In our experience, with a single exception, there has been no overlap in the patterns of the two groups.

DISCUSSION

It is now possible to describe in quantitative fashion the distribution of acid and alkaline phosphatase in the leucocytes of the normal subject and those of the subject with neutrophilic leucocytoses or myelocytic leucemia. The established patterns of high alkaline-normal acid phosphatase activity in leucocytosis and that of low alkaline-high acid phosphatase activity in chronic myelocytic leucemia immediately raise many intriguing and fundamental questions about their significance. What is the role of phosphatase in the metabolism of the leucocyte? Can the changes in leucocyte phosphatase which occur, for example, in infection be correlated with the increases which occur in tissue phosphatase activity found histochemically in stressed animals?[44] Is there any validity to the teleological argument that an increase in the phosphatase contained in a cell represents a metabolic response to stress which is purposefully intended to accelerate the machinery of resistance? Do the metabolic alterations in leucemic cells bear any relation to the vague increased susceptibility to infection observed in the patient with chronic myelocytic leucemia? Can a decrease in cell phosphatase be related to fundamental properties of neoplastic cells in which, for example, growth and proliferation may outrun the ability of the cell to provide its progeny with a full or normal quota of metabolic equipment, the result being that other pathways of metabolism must be invoked to provide energy and nutrition?

The physiologic significance of phosphatase is fairly well understood in certain situations such as calcification and the renal tubular transport of sugar. However, it is safe to say that as yet no unified theory can be presented to explain the nature and specific importance of this ubiquitous enzyme. As has been emphasized by Cohen and McGilvery,[45] the designations "acid" and "alkaline" phosphatase actually depend on the pH levels arbitrarily used by the investigator. Newman and co-workers,[46] studying the distribution of alkaline phosphatases by histochemical techniques, found three classes of phosphatases acting at pH 9.2, distinguishable by their localization within the cells and their affinities for different substrates. The literature pertaining to phosphatase[47, 48] provides abundant evidence on the differences between the various phosphatases which depend upon their pH optima, solubility, substrate specificity, site of origin, activators, and inhibitors. The data of the present study permit no

conclusions about the nature and role of the phosphatase in the leucocyte's metabolism. Rather, there has been shown a decided difference in activity between leucocytes of health and disease which, it is hoped, can be integrated with other known metabolic parameters to provide a basis for further experiments on the energy metabolism of normal and abnormal leucocytes.

To date, studies in various laboratories have shown marked differences in the leucocyte content of glycogen, zinc, histamine, and sulfhydryl groups in health, and in the presence of leucocytosis and myelocytic leucemia. The patterns which have been established are summarized in Table IV.

It should be emphasized that these data, in addition, provide the basis for new diagnostic techniques and classifications of leucocytic diseases. It may be possible eventually to discern from metabolic data of this sort, information about the ultimate nature of the abnormality, its stage of development, prognosis, and clues to its successful treatment. According to ordinary clinical criteria, one could not but suspect the diagnosis of chronic myelocytic leucemia in Patient W. H. described. However, his divergence from all other cases of leucemia so

TABLE IV. A SUMMARY, TAKEN FROM THE LITERATURE, OF CHEMICAL AND ENZYMATIC PATTERNS OF THE LEUCOCYTES OF LEUCOCYTOSIS AND CHRONIC MYELOCYTIC LEUCEMIA. THE COMPARATIVE EXPRESSIONS REFER TO THE CONTENT OF EACH LEUCOCYTE AS COMPARED TO THE NORMAL LEUCOCYTE

	LEUCOCYTOSIS	CHRONIC MYELOCYTIC LEUCEMIA	REFERENCE
Glycogen	?	Increased	9
Zinc	?	Decreased	25
Histamine	Decreased	Increased	8
-SH groups	?	Increased	6
Alkaline phosphatase	Greatly increased	Decreased	
Acid phosphatase	Normal	Normal to high	

far tested in both leucocyte phosphatase and histamine strongly suggests the possibility of some heretofore unrecognized subgroup or deviation which only further observation and metabolic studies of numerous patients can clarify. Conversely, Patient Y. A. (Table III) represents an instance of mild leucocytosis associated with mild nonspecific symptoms and unaccompanied by any diagnostic clinical and morphologic characteristics of leucemia, yet who has the suggestive pattern of leucemia in both the leucocyte phosphatase and histamine. Again, only prolonged observation can confirm the strong suspicion aroused by these findings.

The main purpose of studies of this nature should clearly be the pursuit of fundamental information concerning the wide metabolic differences between the similar and sometimes morphologically indistinguishable leucocytes of leucemia and those of normal subjects or subjects with physiologic leucocytosis. The usual clinical diagnostic criteria are, of course, more than adequate and will surely remain so except in the occasional case. The questions posed herein cannot now be given definite answers; instead they must await much necessary future work.

SUMMARY

Quantitative studies on the alkaline and acid phosphatase content of isolated leucocytes have been reported. Striking differences in the levels of phosphatase activity were noted between the leucocytes of health, leucocytosis, and chronic myelocytic leucemia. In general, there were found high alkaline-normal acid phosphatase levels in leucocytosis and low alkaline-normal to high acid phosphatase levels in chronic myelocytic leucemia. The data on alkaline phosphatase in leucocytosis and chronic myelocytic leucemia did not overlap, with the exception of one patient.

The literature on the chemical constituency of leucocytes has been reviewed and emphasis has been placed on the emerging patterns of chemical and enzymatic activity in the leucocytes of health, leucocytosis, and leucemia. It is felt that the chief value of such data lies in its fundamental implications regarding the nature of the abnormality and, in addition, that it provides a basis for new diagnostic techniques.

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THE MORPHOLOGIC DISTRIBUTION OF INTRAVENOUSLY INJECTED FATTY CHYLE AND ARTIFICIAL FAT EMULSION IN RATS AND DOGS

RAYMOND G. MURRAY, PH.D.,* AND SMITH FREEMAN, M.D., PH.D.
CHICAGO, ILL.

THIS report compares the immediate fate in rats and dogs of intravenously injected artificial olive oil emulsion with that of fatty dog chyle injected in similar quantity and manner. It was found that by ten minutes after injection, the fat of emulsion always, but of chyle never, was concentrated in the capillaries of the lung and the marginal zone of the splenic red pulp. The Kupffer cells of the rat liver usually did not contain significant amounts of fat. Chyle fat enters the liver parenchymal cells directly and rapidly in dogs and probably also in rats. There was some evidence that the artificially emulsified fat entered the liver cells more slowly and perhaps indirectly by way of the Kupffer cells.

Since emulsions of fat possessing a high degree of stability and apparently low toxicity have been prepared by several investigators[1, 2] and are being administered to patients[3, 51] knowledge of the mechanism by which the body handles this fat is highly desirable. Any attempt to evaluate the normality of the body's handling of fat emulsions is handicapped by insufficient knowledge of normal fat metabolism. A comparison of histologic evidence of the fate of fatty chyle in the body with that of an artificial emulsion may give some evidence of the extent to which the latter actually simulates chyle. Under the conditions of the experiment, it cannot be stated whether or not the handling of either substance can be taken as representing the normal train of events that occur during fat absorption.

MATERIAL AND METHODS

Male and female Sprague-Dawley rats ranging in weight from 210 to 510 grams were used. They were maintained on a diet of rat pellets supplemented with bread and horse meat, except that the meat was excluded three to ten days prior to injection. The dogs were mongrels kept in the laboratory for variable times, usually a week or more, and maintained on a diet of bread and horse meat.

The emulsions were prepared from olive oil stabilized with Asolectin,† Span 20,‡ and sodium deoxycholate, with 5 per cent dextrose added. The ingredients were blended in a Waring Blendor and homogenized with a Cherry-Burrell viscolizer§ essentially in the

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*Present address: Department of Anatomy, Indiana University School of Medicine, Bloomington, Ind.

†A purified soya bean phosphatide, product of Associated Concentrates, Inc., Atlanta, Ga.

‡Surface active agent, produced by the Atlas Powder Company, Wilmington, Del.

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manner described by Meng and Freeman.[2] Samples under the microscope showed a nearly uniform particle size in the vicinity of 1 μ with perhaps one drop in several high-power fields measuring 3 to 5 μ. These emulsions were stable for several months but were never used longer than two weeks after preparation.

Chyle was collected under Nembutal anesthesia from the thoracic duct for a six- to ten-hour period. From three to five hours prior to the operation, the dogs received about 4 Gm. per kilogram of olive oil by stomach tube. Glass cannulae were found most satisfactory for collection, but plastic tubing was used successfully in one instance. In all chyle experiments except the earliest tests on rats, the dogs were maintained on 50,000 units of crystalline penicillin injected subcutaneously every four hours during the operating and collecting period with about 10,000 units per 100 ml. of chyle added to the collecting flasks. These flasks were sterilized beforehand and placed immediately in the icebox after one-half to two hours' flow of chyle had been collected. Just before injection, the chyle was gently expressed from the clot through several layers of gauze into a beaker containing about 1 drop of heparin (Liquaemine, Roche-Organon) for each 40 or 50 ml. of chyle. A sample of this mixture was analyzed for total fatty acids by the method of Smith and Kik.[4] The containers and injection apparatus were sterilized for the dog experiments and injected dogs received the aforementioned dose of penicillin.

The rats were injected by tail vein after warming the tail for one to two minutes in water at 46° C. to 48° C. The artificial emulsion or chyle was allowed to come to room temperature before injection and was administered by syringe at a rate of 5 ml. in about two minutes. With the dogs, a glucose infusion was started at a very slow rate and when the preinjection biopsy was completed the emulsion or chyle was substituted, being then administered at a rate such that 150 to 300 ml. were given in fifteen to thirty minutes.

The rats were biopsied without aseptic precaution under ether anesthesia through a midline incision. The left lobe of the liver was exteriorized, a ligature placed around its base, and excised. The incision was closed in two layers with continuous silk. Within half an hour these rats appeared only moderately active but showed no other obvious ill effects. It was noted, however, that injections in the tail veins were more difficult in these animals due to the relative failure of the veins to fill in response to warming. An interval of three to four hours was always allowed between biopsy and injection.

The dogs were operated upon with sterile precautions under 15 to 20 mg. per kilogram of intravenous Nembutal anesthesia, supplemented by an earlier injection of approximately 0.15 mg. of atropine sulfate and 2.5 mg. of morphine sulfate per kilogram. The abdomen was opened in the midline, a punch biopsy taken with a trocar through a lobe of the liver, and a wedge-shaped slice cut from the margin of the spleen. Hemostasis of the spleen was effected with sutures and Gelfoam,* while in the liver punches a plug of Gelfoam was sufficient. The fat was then administered, the biopsy procedure repeated, and the incision closed in three layers with continuous silk suture. For subsequent biopsies, the residual Nembutal anesthesia was supplemented with ether.

All tissues for sectioning were placed at once in 10 per cent Formalin neutralized with $CaCO_3$. Frozen sections were prepared in most cases within a week and in many cases on the day following autopsy. The sections were stained for fat with oil red O[5] by the supersaturated isopropyl alcohol technique[6a] with the addition of rinses in 60 per cent and 40 per cent isopropyl alcohol after staining. Lillie-Mayers acid alum hematoxylin[6b] was used as a counterstain, and the sections were mounted in Kaiser's glycerin jelly.[7] In the earliest work the tissues were embedded in 20 per cent gelatin for sectioning, but there was evidence of leaching of fat in these sections so the majority of tissues were cut without embedding.

OBSERVATIONS

None of the organs of rats at autopsy displayed any significant gross changes with the exception of the liver and spleen which were engorged at early intervals

*A product of The Upjohn Company.

after injection. In the dog liver a definite pale mottling which accentuated the lobular markings on the surface of the liver became apparent within ten to fifteen minutes after the beginning of the injection of either chyle or emulsion. It was more marked and persisted longer after emulsion than after chyle. The spleens of all dogs were greatly distended with blood by the anesthesia until several hours after injection when the organ shrank again as the effect of the anesthetic diminished.

Description of the microscopic findings in rats will be divided into three series, the first dealing with young female rats weighing 220 to 270 grams and killed at intervals from ten minutes to twenty-four hours after injection of emulsion or chyle, with suitable untreated controls. In the second series, a liver biopsy was taken from about half of the rats three to four hours prior to injection of chyle or emulsion, and in the third series, olive oil was fed by stomach tube. A summary of the rats in the first series is given in Table I.

Microscopic Observations on Intravenously Injected Rats.—The organs routinely examined in the first series were liver, spleen, kidney, lung, heart, thymus, cervical lymph node, stomach, duodenum, ileum, and adrenal. In the frozen sections stained with oil red O, the injected fat stained an intense, brilliant red color when in large globules and a somewhat darker but no less intense red when in fine granular form.

TABLE I. YOUNG FEMALE SPRAGUE-DAWLEY RATS USED IN FIRST SERIES

		TREATMENT			
		CHYLE	EMULSION	EMULSION	UNTREATED
Per cent fat		3.8	10.0	3.8	—
I. V. ml. per kg.		20.0	10.0	20.0	—
Grams fat per kg.		0.76	1.0	0.76	—
Weight range (Gm.)		230-260	220-270	220-260	220-270
Number of rats at each interval	10 min.	2	2	2	4
	30 min.	2	2	2	4
	2 hr.	2	2	2	3
	6 hr.	2	2	2	4
	24 hr.	2	2	2	4
Total		10	10	10	19

In the descriptions that follow, the term "chyle" refers always to the fatty chyle collected from the thoracic ducts of dogs; the term "emulsion" refers to the artificial olive oil emulsions, the preparation of which has been described briefly. The chyle used in the first series had been collected the previous day, and under the microscope the contained fat was seen to be composed of uniformly fine granules less than 1 μ in diameter. Of the organs examined none showed significant accumulations of fat at any of the intervals sampled after injections of this material equivalent to 0.76 Gm. of fat per kilogram. At the ten- and thirty-minute intervals there was evidence of fine fat granules in the blood in all organs, but only within the vessels. In contrast, after injections of emulsion equivalent to 1.0 Gm. of fat per kilogram, the lung and spleen showed marked accumulations of fat. The Kupffer cells of the liver were free of fat and the content of the liver parenchymal cells varied within the limits

seen in untreated rats. Other organs did not differ significantly from the untreated specimens with the exception of the fat contained in the vessels. The appearance of spleen and lungs will be described in more detail.

Spleen: At the earliest interval after injection of emulsion (ten minutes), the region of the red pulp immediately adjacent to the white pulp was heavily infiltrated with irregular partly coalescent globules of fat (Fig. 1). This zone, the Knötchenrandzone of Weidenreich,[8] will hereafter be referred to simply as the marginal zone. Although exact localization of the fat was difficult because of the indefinite limits of the phagocytes and a tendency for the fat to be displaced during preparation of the sections, it appeared that much of the fat at this early interval was between rather than within the macrophages. However, the fat deposit in the marginal zone was sharply demarcated both from the white pulp and from the remainder of the red pulp. In these two latter regions there were no red-stained deposits except occasional dark red-brown granules in the macrophages. Similar deposits were seen in the spleens of untreated rats in this group.

At thirty minutes there was an even heavier massing of fat in the marginal zone with little evidence of concentration within the macrophages. The periphery of the marginal zone concentration was irregular in contrast to the relatively sharp line of demarcation seen here at ten minutes, and a few of the macrophages of the red pulp contained bright red inclusions. The white pulp was still free of stainable fat.

At two hours there was still a great deal of fat which, though largely in the marginal zone, was present in the central red pulp in larger amounts than formerly. Most of the fat in all regions was in macrophages.

At six hours there was much more fat in one of the two specimens than in the other but much less in either than at thirty minutes. The fat was largely in the central red pulp and entirely within macrophages, predominantly those of the cords.

At twenty-four hours two of the three spleens were fat free and the third contained some fat in the macrophages of the red pulp. There were numerous small foci of mononuclears in the liver of this rat which might justify its exclusion from the normal picture.

Lung: Ten minutes after injection, the capillaries of the lung contained large numbers of fat droplets, mostly about 3 to 5 μ in diameter (Fig. 2). Alveolar spaces and macrophages in the alveolar walls contained no fat. At thirty minutes the capillaries were free of fat, but there were occasional small groups of macrophages in the alveolar walls containing moderate amounts of fat in large droplets. These fat-filled macrophages were found at six and twenty-four hours also. No fat reached the hilar lymphatic tissue nor was there a significant amount of fat in the air spaces.

Liver: Because the interpretation of the liver sections was clouded by the variations in fat content of untreated livers, another group of rats, in this case Sprague-Dawley males ranging from 360 to 510 grams, was injected approxi-

mately four hours after liver biopsy with chyle or emulsion, plus an equal number of unbiopsied rats for comparison. Table II summarizes the animals killed at the two intervals.

Liver, spleen, and lung only were examined in this group. The chyle used for these injections was collected three days previously. It had been refrigerated during that time, and on microscopic examination appeared exactly the same as the fresher material used in the first series.

TABLE II. OLDER MALE SPRAGUE-DAWLEY RATS USED IN SECOND RAT EXPERIMENT

	TREATMENT				
	CHYLE	CHYLE	EMULSION	EMULSION	NONE
Per cent fat	4.4	4.4	5.0	5.0	—
Ml. injected per kg.	20.0	20.0	20.0	20.0	—
Grams fat injected per kg.	0.9	0.9	1.0	1.0	—
Biopsy	Yes	No	Yes	No	Yes
Weight range (Gm.)	400-510	430-500	360-460	370-460	370-480
Number of rats 10 min. at each interval	2	5	3	3	3*
1 hr.	3	3	3	2	

*Autopsy four hours after biopsy.

The appearance of the lungs did not differ appreciably from the earlier series. The spleens after emulsion injections, either with or without biopsy, differed only slightly in that a small amount of fat appeared at the earliest interval in the macrophages of the cords of the red pulp in addition to the masses in the marginal zone. The spleens of rats that received chyle showed distinct differences from previous chyle injections. At ten minutes with or without previous biopsy, there was concentration of fat in the cords of the red pulp, but none in the sinuses, none in the marginal zone, and none in the white pulp. This fat was in finely divided form in contrast to larger drops characteristic of the deposition of emulsion. It was not entirely within the macrophages of the cords at ten minutes, but much was still present at one hour, then entirely in the macrophages. The total amount of fat seen here was quite small compared with that seen after injections of emulsion.

The liver Kupffer cells, as in the previous experiment, did not concentrate the fat after injections of chyle. In two of the three rats without biopsy killed

Fig. 1.—Spleen of a rat which ten minutes previous to sacrifice received by tail vein 1.0 Gm. per kilogram of fat in the form of artificial emulsion. The fat is concentrated in the marginal zone of the red pulp. (Oil red O and hematoxylin. ×120.)

Fig. 2.—Lung of a rat which ten minutes previous to sacrifice received by tail vein 1.0 Gm. per kilogram of fat in the form of artificial emulsion. (Oil red O and hematoxylin. ×115.)

Fig. 3.—Peripheral zone of a liver lobule from a rat which ten minutes before sacrifice received by tail vein 1.0 Gm. per kilogram of fat in the form of artificial emulsion. A branch of the portal vein is shown. Note that the Kupffer cells are virtually free of fat. (Oil red O and hematoxylin. ×300.)

Fig. 4.—Peripheral zone of a liver lobule from a rat at the same interval after the same dose as that of Fig. 3. However, four hours before injection a lobe of this liver had been excised under ether anesthesia. Fat is concentrated primarily in the Kupffer cells near the periphery of the lobule. (Oil red O and hematoxylin. ×300.)

Fig. 5.—Spleen of a dog immediately after a twenty-minute intravenous infusion of fatty chyle (approximately 0.65 Gm. per kilogram total dose). Fat is concentrated in the sheathed arteries and is absent from the margins of the white pulp. (Oil red O and hematoxylin. ×110.)

Fig. 6.—High magnification of one of the sheathed arteries from the spleen shown in Fig. 5. The central lining cells are free of fat while the concentrically arranged reticular cells are heavily loaded. (Oil red O and hematoxylin. ×1440.)

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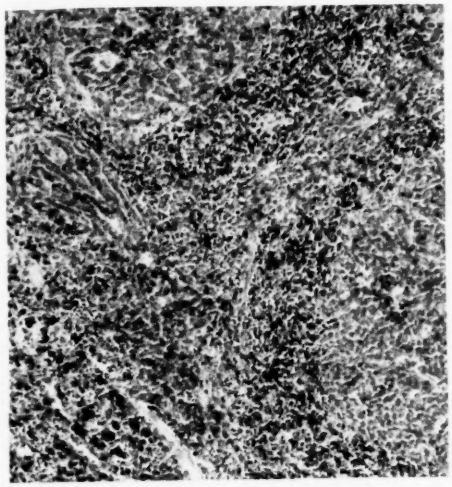
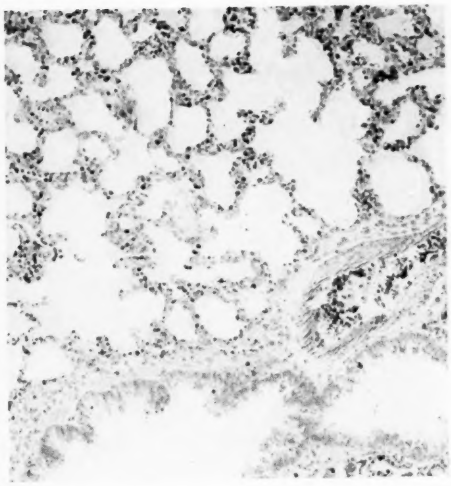


Fig. 1.



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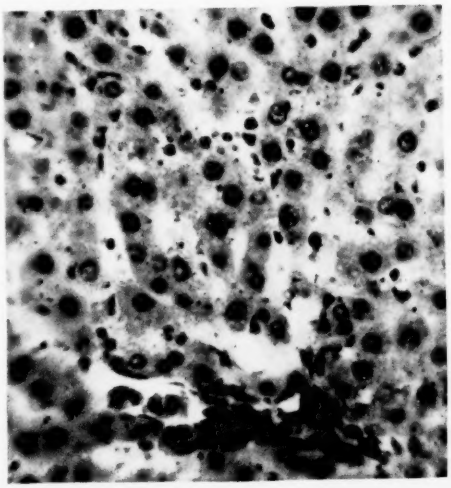

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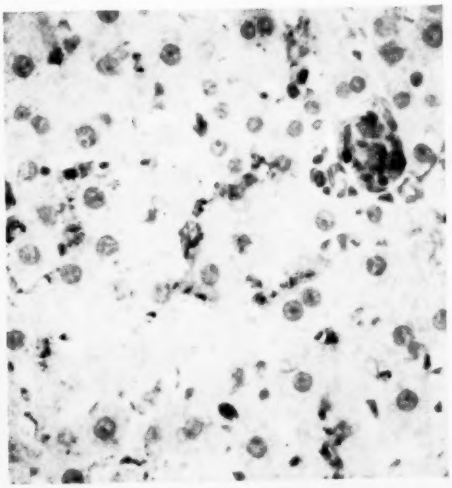

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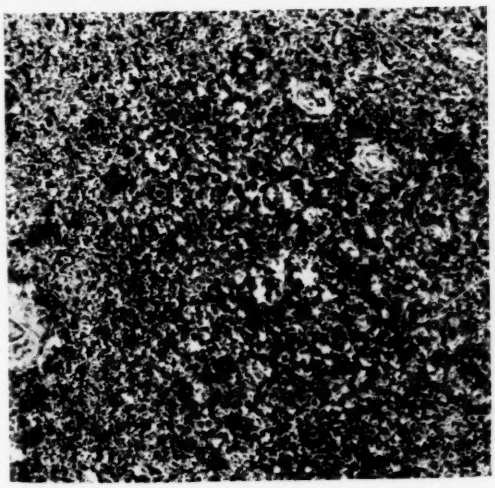

Fig. 5.

Fig. 6.

ten minutes after injection, there was a high content of fine fat granules in the sinusoids, but this appeared to lie along the margins of the liver cells and was associated with a considerable content of fat in the contiguous peripheral cytoplasm of the liver cells. The biopsied rats showed virtually no fat in the sinusoids. Reliable judgments of the fat content of the liver cells were partially vitiated in this series by the presence, in a small number of biopsied but otherwise untreated rats, of changes in fat content nearly as extensive as those seen after injection. At one hour after injection of chyle, with or without biopsy, both sinusoids and Kupffer cells were virtually free of fat.

In the liver after injections of emulsion in biopsied rats, almost all the fat present at ten minutes was in the Kupffer cells. The majority of the fat was toward the portal side (Fig. 4) and most of this remained at one hour. This was in sharp contrast to the absence of fat in the Kupffer cells in the early series and in the unbiopsied rats of this series (Fig. 3). At one hour there was a moderate amount in liver cells also, probably more in those cells near loaded Kupffer cells than elsewhere. In general, however, the amount of fat in liver cells was not appreciably greater than that seen in biopsied, uninjected rats.

Rats Given Olive Oil by Stomach Tube.—Table III summarizes the intervals sampled. Lung, spleen, and liver only were examined, the first two of which showed no trace of the ingested fat. It was in the rather large and uniform group of control rats in this series that the great variation in fat content of the untreated liver was most clearly shown, thus precluding any conclusions regarding the extent to which the recently ingested fat had entered the liver cells. There was none in the Kupffer cells, however, either in the experimental or the control animals.

TABLE III. FEMALE SPRAGUE-DAWLEY RATS WEIGHING 210 TO 250 GRAMS GIVEN 3 ML. OF OLIVE OIL BY STOMACH TUBE

INTERVAL (HR.)	NUMBER OF RATS TREATED	NUMBER OF CONTROL RATS*
2	3	3
4	3	3
8	3	3
23	4	4
Total	13	13

*Each of these rats was sham injected by introducing and withdrawing a tube lubricated with mineral oil.

Observations on Dogs.—From Table IV it is clear that the experiments with dogs were limited in number and not uniform with respect to dose, animal weights, or freshness of the chyle used. However, the response of the spleen roughly paralleled the rat experience for both chyle and emulsion. There was the same heavy concentration in the marginal zone at early intervals after emulsion, but chyle fat never was found there. The one distinct difference between rats and dogs was a concentration of fat in the sheathed arteries (ellipsoids) of the spleen, as great after chyle (Fig. 5) as after emulsion. The fat was largely in the reticular cells of the ellipsoids; the lining cells of the central arteriole of the sheath were free of fat granules (Fig. 6). Fat was present in the

ellipsoids at three hours after chyle, gone at six hours after chyle, but still present six hours after emulsion. The amount of fat varied widely in the few specimens, one dog with emulsion showing very heavy deposition and the other only a scattering of granules. No fat remained in any spleen at twenty-four hours.

TABLE IV. SUMMARY OF SERIAL BIOPSY EXPERIMENT ON DOGS

WEIGHT OF DOG (KG.)	MATERIAL	VOLUME INJECTED (ML.)	FAT (%)	FAT (GM./KG.)	LENGTH OF INJECTION (MIN.)	BIOPSY INTERVAL				
						PRE-INJECTION	HOURS AFTER INJECTION			
							0	3	6	24
11.0	Chyle*	300	4.4	1.2	21	x	x	x		x
11.5	Chyle†	240	2.9	0.6	15	x	x		x	x
22.0	Emulsion	200	10.0	0.9	30	x	x		x	‡
23.0	Emulsion	150	10.0	0.65	17	x	x	x		x§

*Collected two days previous to injection.
†Collected one day previous to injection.
‡Dog died in the night after the third biopsy.
§Dog died shortly after the twenty-four hour biopsy.

The pre-injection biopsies of liver were much alike in all four dogs. There was either no fat in the liver cells or a scattering of fine, dark granules about the nucleus in a few cells near the central vein. The bile duct epithelium in each liver was heavily loaded with fat. Sinusoids and Kupffer cells in no case contained significant amounts of fat.

The appearance of the liver after chyle injection differed from that seen in rats. At the immediate postinjection interval in one dog there was considerable fat, almost entirely within liver cells. In contrast, in the operated rats the fat was almost entirely free in the sinusoids. The fat in this dog lay in the margins of the cells and could be clearly distinguished from the darker granules near the nucleus which were present as in the preinjection specimen. The other dog showed similar but minimal accumulation of fat. In neither dog was there appreciable fat in the sinusoids. At later intervals, it was not possible to distinguish the injected fat from that which pre-existed in the liver cells, but in general the fat present was again centrally located in the cells. There was no change in the fat of the bile duct epithelium.

The livers of the two dogs receiving emulsion were similar and resembled those of the rats with previous biopsy. At the earliest interval the fat was almost all in the Kupffer cells, and at three hours still largely remained there. At six hours Kupffer cells at the periphery of the lobules (where fat had previously been found) were empty, but the liver parenchymal cells in this region contained moderate amounts of finely divided, brightly staining fat granules. Elsewhere in the lobules both Kupffer cells and parenchymal cells were free of fat. At twenty-four hours the fat was entirely within liver cells, and toward the center rather than the margins of the cells.

DISCUSSION

Of the many studies on intravenously administered artificial emulsions of fats and lipoids, a few have included examinations of histologic material. The

more pertinent of the latter will be considered briefly. On the other hand, there have been very few studies with injected chyle and no histologic investigations. Oeken,[9] Bauersfeld,[10] and Little and associates[11] reinjected the chyle from patients with chylothorax without apparent toxic symptoms. Rony and Mortimer[12] injected dogs with chyle from other dogs previously fed with egg yolk and found that the fat rapidly disappeared from the blood stream. Berry and Ivy[13] compared, in dogs, the relative rates of disappearance from the blood stream of fat injected in the form of chyle with fat as lecithin-stabilized butter oil emulsion. They found that chyle was cleared from the blood stream more rapidly than the synthetic emulsion.

In spite of inconsistencies between the several groups of rats and differences between rats and dogs, a few relatively reliable observations can be made. The artificially emulsified fat used in this laboratory, when injected intravenously at a relatively rapid rate in relatively large amounts, invariably appeared in the marginal zone of the red pulp of the spleen. Comparably, injected chyle was never seen here although it was found in very limited amounts in the macrophages of the cords of the red pulp. Although Leites,[14] Sato,[15] and others found by analysis that injected artificial emulsions lodge to some extent in the spleen, those who have made histologic sections of organs after emulsion injections either do not mention the spleen[18, 3, 16, 17] or mention only that fat was found in the macrophages of the red pulp.[18, 19, 20]

Between the white pulp sheathing the arteries and the typical red pulp, with its venous sinuses and cords of Bilroth, is a zone which contains mostly loosely arranged medium sized lymphocytes, many reticular cells, and no venous sinuses. From such a description, it would appear that this zone should be included in the white pulp as certain authors have done.[21] However, there is good evidence that this zone has a blood supply separate from that of the white pulp.[22, 23, 24] The majority of evidence regarding the circulation through the spleen seems to suggest that, under certain conditions at least, there is some type of "open" circulation,[8, 25, 26, 27, 28b] and this marginal zone appears to be one region, perhaps the principle one, where blood is poured into the red pulp spaces. This is suggested by the frequency of hemorrhage in this area,[29] and by a predilection for certain intravenously injected substances to concentrate here.[24, 28a, 31, 32, 39] Maximow[28a] considers this zone part of the red pulp as do Maximow and Bloom.[28b] The presence of fat concentrations in this region can be looked upon as, on the one hand, evidence that the fat is treated to some extent as a foreign material, and on the other hand as evidence compatible with the "open" circulation theory, particularly since at early intervals much of the fat appears to lie outside the cells. However, several authors[20, 33, 34] report injections of particulate matter which, though lodging in the red pulp, do not concentrate in the marginal zone. There must be many factors which result in different distribution under different physiologic conditions. MacKenzie[27] stressed the importance of contractions of the spleen under certain conditions altering the circulatory path. When the spleen is highly contracted, a functionally "closed" type of circulation might exist, in which case the marginal

zone might not be heavily contaminated with circulating particles. The rat spleen contains only small amounts of smooth muscle so its contractile ability may be limited. We have no data on the state of contraction in the rats, but at the time of injection, the highly muscular spleens of the dogs were quite large and engorged with blood. It is possible that investigators who have failed to note deposits of fat in the marginal zone were dealing with highly contracted spleens, although we have no direct evidence that in such spleens the fat would not lodge in the marginal zone.

The presence of fat in the ellipsoids (sheathed arteries) has been noted previously by Krause[35] but has not been reported in animals injected with fat. Robinson[36] and Solnitsky[37] report that the ellipsoids are an early site of deposition of injected particulate matter. Tait and Cashin[30] and Mills[31] found not only that the ellipsoids are first to show accumulation, but that they may be stained with doses too low to be concentrated in the marginal zone. Our results with chyle agree with this observation; but, in the case of emulsion, in one dog the marginal zone was rich with fat while there was only a trace in the ellipsoids. In the rat spleen, fat was never concentrated in a manner suggestive of the existence of ellipsoids in this species. In general, our results would seem to indicate a fundamental difference in the mechanism by which the two regions accumulate fat particles. In this connection we noted that the fat in the ellipsoids apparently was largely within the reticular cells even at the earliest interval, while much of the fat in the marginal zone tended to lie outside the macrophages at early intervals. This would be consistent with the interpretation of other authors[31, 36] that the blood passes out rapidly through openings in the vessel within the ellipsoid and between the densely packed reticular cells. Particles to remain here would almost have to be actively ingested by the cells. In the marginal zone, however, where the structure is looser and the flow of blood perhaps less rapid, simple mechanical factors might operate to cause an accumulation of fat.

We have seen that the fat leaves the marginal zone in the course of a few hours and moves out into the region of the splenic cords. This might explain the absence of fat in the marginal zone if only late intervals were studied[20] or if the fat were given over a long period of time.[18, 19]

It might be said in summary of our findings with respect to the spleen that they are most easily interpreted on the basis of an "open" type of circulation and suggest that the artificial emulsions, and to some extent the fatty chyle also, are handled by the spleen much like certain other foreign materials.

The variability of fat content in liver parenchymal cells under essentially stable conditions, in our experience, presents a formidable obstacle to the interpretation of events occurring in this organ. In our sections, the content of oil red O-stained material in liver cells varied as widely in untreated rats as in any of the experimental rat series. This was true of rats which had had no meat as well as those which had had meat up to a few days before sacrifice. Although many conditions are known to affect the fat content of the liver,[38] these

were either constant in our material or could not be correlated with changes in fat content. We found, as had others,[39, 40, 41] that starvation does not clear the liver cells of fat. The procedure of liver biopsy, previously used in fat studies by Macchiarulo[42] and by Nomura[17] did not answer this objection in the case of the rats, for the samples of liver taken at autopsy of biopsied but otherwise untreated rats showed a different fat content in liver cells than had the biopsy specimens secured several hours earlier. In the dogs, however, serial punch biopsy gave more interpretable results, probably because the interval between the control and the early experimental biopsies was short. Here again, in biopsies taken six hours or more after the control biopsy, the source of stained fat could not be determined.

The Kupffer cells, in contrast to the liver parenchymal cells, never contained significant amounts of fat in any untreated specimens. Fat in the Kupffer cells was, therefore, good evidence that these cells were taking up the injected material. The fact that the Kupffer cells contained fat in some series and not in others is probably related to the fact that in all cases where fat was found, either the animal was being or recently had been operated on under general anesthetic. It might be that impairment of the circulation or a more direct effect of the anesthetic on the Kupffer cells was responsible for these differences. When fat was seen in the Kupffer cells, it was more prominent toward the periphery of the lobules, in agreement with Chalatow.[43]

There is no general agreement in the literature as to whether injected fat emulsions are phagocytized by the Kupffer cells. McKibben and associates[16] and Dunham and Brunschwig[18] found fat in the Kupffer cells after daily infusions of emulsions. Chalatow,[43] Saxl and Donath,[44] and Jaffé and Berman[45] reported fat in the Kupffer cells after single injections of emulsions, while Nomura[17] and Nachlas and associates[16] also after single injections found none. These differences may reflect, in part, differences in the fat or emulsifier used. Our results with unanesthetized animals are in accord with the last two observations. The first two investigations involving daily injections over long periods constitute a different case. It may be significant in this connection that the macrophages of the main portion of the splenic red pulp in our material did not contain fat at early intervals. Taken together these facts might suggest that the concentration in the marginal zone of the spleen represents not so much a phagocytosis of circulating material (in which case one would expect to find it in the Kupffer cells also) as a stagnation largely from mechanical causes. This in turn is probably related to the agglutination and coalescence of the fat particles which can be shown to occur quite rapidly when artificial emulsion is introduced into rat blood either in vivo or in vitro. Such agglutination does not occur with chyle, and with emulsion it is much less pronounced in dog and human blood than in rat blood. It may be that the differences between chyle and emulsion are largely related to the different particle size which results from this agglomeration of the particles of the artificial emulsion.

The importance of the Kupffer cells in the transport of fat into the parenchymal cells has been stressed by Jaffé and Berman.[45] Our work is in accord with their findings only in the case of anesthetized animals where there was,

after injections of emulsions, massive accumulation of fat in Kupffer cells and some suggestion that the fat was passed on to the parenchymal cells. The injections of chyle, on the other hand, demonstrated that fat administered in this form may enter the parenchymal cells directly without involving the Kupffer cells.

Tracing injected fat through the body, particularly through an organ which has a significant and variable fat content such as the liver, would be greatly facilitated if a reliable labeled fat could be used. Jaffé and Berman[45] were able to trace the injected fat by staining with Nile blue sulfate. We did not use this stain, but its reliability and specificity are not beyond question. Isotope-labeled fat would give information as to general distribution of the fat, but at the cellular level the resolution of the autoradiographs is inadequate for exact localization. Several investigators[15, 46, 47] have used iodine-labeled fat for determination of distribution of injected fat either by chemical analysis for the iodine or by x-ray study of the animals. We have made preliminary studies with two rats by roentgenograms after injection of emulsified Lipiodol which showed a definite increased density in the spleen but a hardly significant change in the liver shadow. By refinement of the technique, it is possible that useful information could be gained by this method. Other rats, which had been injected ten minutes previously with emulsified Lipiodol, were sectioned in the usual manner and the slides treated with silver nitrate. Deposits of darkened granules were seen in the spleen in the same location as could be demonstrated on serially adjacent sections with the oil red O. There were no blackened granules in the liver either in the Kupffer or parenchymal cells. The absence of blackening cannot be considered conclusive evidence that none of the injected fat was in the liver, for the iodine might have been separated from the fat. It would appear that even a combination of all the foregoing methods leaves much to be desired.

The role of the lung in retaining injected fat can be determined more reliably than that of the liver for there is normally only a small quantity of oil red O-stained material scattered in single or small groups of macrophages in the alveolar septa. At ten minutes after injection of emulsion, however, the capillaries are crowded with globules of fat ranging in size up to that of the capillary bore. This large size is apparently due to the agglutination and coalescence of fat particles mentioned. At thirty minutes and subsequent intervals this fat is gone from the lungs except for a moderate scattering of globules in occasional septal macrophages. This rapid passage of fat through the lungs does not support the view of Roger and Binet[48] and others that the lung plays an important role in fat metabolism. The fact that previous investigators have not described such massive infiltration of the lung may mean that there was less agglutination of their emulsions or they failed to examine the lung immediately after injection. The retention of small amounts of fat in the septal macrophages might be the source of the granulomatous lesions in rat lungs after injections of emulsions as reported by Collins and associates.[49]

The lack of stainable fat in the kidney tubules is evidence that the fat is not excreted by this route. This finding is in accord with all previous results

except those of Dunham and Brunschwig[18] who found fatty deposits in the kidney tubules and fat in the urine by analysis. The considerably larger doses he gave, or the greater toxicity of the stabilizer he used, might account for this difference.

We believe the results in all organs taken together indicate that the synthetic fat emulsion, while appearing nearly as finely divided as the chyle, is not handled by the body in exactly the same manner; it is treated much like a foreign material. There is evidence that the fat of chyle enters the liver cells directly, and that the fat of emulsion enters the liver cells somewhat more slowly and possibly indirectly by way of the Kupffer cells. This last might be taken as evidence that a significant portion of the artificially emulsified fat is readily utilized. This finding is in accord with evidence for utilization of injected fat obtained by studies with radioactive carbon.[50]

Further studies should be made to test the significance of the many variable and poorly controlled factors in the work here reported. The influence of heparin on the rate of clearance of fat from the blood stream and on the pattern of distribution of chyle and emulsion should be investigated. The effects of various lengths of injection time and ratios of volume to grams of fat should be tested. The agglutination and coalescence when emulsion meets blood must be avoided either by seeking a species in which it does not occur or by developing an emulsion which is free of this defect. The rat appears to be a particularly poor choice in this respect. Greater numbers of dogs should be used. Differences introduced by varying lengths and conditions of storage should be studied, and the fate of a variety of emulsified lipids should be tested.

SUMMARY

The distribution of fat in certain organs of rats and dogs was studied primarily by frozen sections stained with oil red O, at intervals from ten minutes to twenty-four hours after rapid intravenous administration of 0.6 to 1.2 Gm. per kilogram of olive oil in emulsified form. This was compared with the distribution of fat following similarly injected fatty dog chyle as well as that of olive oil administered by stomach tube.

Olive oil in the amount of 2 ml. given by stomach tube to rats could not be identified in spleen, liver, or lungs.

The fat of injected artificial emulsions always appeared by ten minutes after injection in large amounts in the marginal zone of the splenic red pulp. In contrast, the fat of dog chyle was never concentrated here.

Within ten minutes after injection of chyle, fat appeared in the parenchymal cells of the liver of dogs; at this interval after artificial emulsion, fat was concentrated in the Kupffer cells. The evidence from rat livers was clouded by variable fat content in the livers of untreated rats. Kupffer cells of rats contained fat only in recently anesthetized animals which had received emulsion; there was some evidence that this fat was passed on to the parenchymal cells. Chyle fat was never concentrated in the Kupffer cells.

Lung capillaries of rats were packed with fat ten minutes after injections of artificial emulsion, but at subsequent intervals only scattered droplets re-

mained in the septal macrophages. Chyle fat was never concentrated in the lung.

Fat did not appear in significant quantities outside the blood vessels in cervical lymph nodes, kidney, heart, thymus, stomach, duodenum, or ileum after injections of either chyle or emulsion.

The sheathed arteries of the dog spleen were sites of a striking concentration of fat in dogs receiving either emulsion or chyle. No concentration of fat could be detected in the rat spleen that would correspond to sheathed arteries.

Possible causes of the considerable variation in results are discussed and the need for a labeled fat for future experiments is indicated.

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TOLERANCE OF HUMAN BEINGS TO INTRAVENOUS INFUSIONS OF FIFTEEN PER CENT INVERT SUGAR*

JACOB J. WEINSTEIN, M.D., F.A.C.S.
WASHINGTON, D. C.

OUR interest in invert sugar followed a search for a parenteral carbohydrate that is readily retained by the body and that can supply a large number of calories per unit of time in a moderate amount of fluid. Invert sugar is a hydrolyzed product of sucrose and consists of equal moles of D-glucose (dextrose) and D-fructose (levulose). Solutions of invert sugar are crystal clear, are stable at room temperature, can be heat sterilized, and are both nonpyrogenic and nonanaphylactic.[1]

We have recently reported the tolerance of human beings to both 50 and 100 Gm. of invert sugar. When 50 Gm. of invert sugar are administered at a rate of 1.5 Gm. per kilogram per hour, or in thirty-four minutes, in either 500 c.c. of distilled water or in 1,000 c.c. of physiologic saline, there is an average retention of 99.2 per cent of the 50 Gm. infused.[2, 3] In the report on 100 Gm. of invert sugar in 1,000 c.c. of water at a similar gram per kilogram rate, but which required sixty-eight minutes for the total infusion of the 100 Gm. of invert sugar, 98.4 per cent was the average amout of sugar retained.[4]

As part of our investigation of the tolerance of human beings to parenteral invert sugar, we studied the effect on the blood and on the urine of 150 Gm. of invert sugar dissolved in 1,000 c.c. of water, administred as a single injection at a controlled rate of 1.5 Gm. per kilogram per hour. This paper presents our observations. Because an average of 96.4 per cent of the 150 Gm. of sugar injected in eighty-nine minutes is retained by the body, we have concluded that 15 per cent invert sugar is readily and almost completely assimilated by normal adults when injected rapidly by vein.

PROCEDURE AND METHODS

A group of twenty-one adults were each given a single controlled intravenous injection of 15 per cent invert sugar in 1,000 c.c. of water at a rate of 1.5 Gm. per kilogram per hour. The subjects were patients in the general surgical wards of the Gallinger Municipal Hospital. They were all considered normal because none were in the immediate postoperative period, all were on regular house diets, all were active about the wards, and each had normal urine analyses, normal blood sugar levels, and normal temperatures. A review of the diagnoses for these patients indicates that many had minor surgical illnesses or were recuperating from previous operations done many days before the tests.

The following plan was carried out for each test: (1) The weight of each patient was determined in the morning before the test and the rate of administration was calculated from

From the Department of Surgery, George Washington University School of Medicine and Gallinger Municipal Hospital.

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this. (2) Breakfast was eaten by each subject approximately two hours before the infusion. (3) Urine was collected by voluntary voiding before the test, immediately after the infusion, and one, two, and three hours after completion of the infusion. (4) Each urine specimen was tested for specific gravity reaction, grams of total reducing substance by Benedict's quantitative test,[5] and grams of fructose by the procedure of Roe.[6] (5) Blood samples were drawn from the arm opposite that used for the infusion, before beginning the infusion, at the end of 500 c.c., after 1,000 c.c., and one, two, and three hours following the infusion. (6) Each blood sample was analyzed for total reducing substance by the method described by Folin[5] and the blood fructose content was determined by the modified resorcinol procedure

SUGAR LOSS WITH 15% INVERT SUGAR IN WATER AT 1.5 gm./kg./hr.

NUMBER OF CASES	AGE YEARS	WEIGHT KG.	GRAMS REDUCING SUBSTANCE EXCRETED IN URINE	GRAMS FRUCTOSE EXCRETED IN URINE	PER CENT REDUCING SUBSTANCE EXCRETED	PER CENT FRUCTOSE OF TOTAL REDUCING SUBSTANCE EXCRETED	PER CENT INVERT SUGAR RETAINED
1	47	71	1.82	025	1.21	13.74	98.79
2	54	72	0.52	0.11	0.35	21.35	99.65
3	64	86	2.3	0.09	1.53	3.9	98.47
4	65	65	3.3	0.17	2.2	5.3	97.8
5	50	93	5.07	0.32	3.38	6.31	96.62
6	49	70	7.24	0.48	4.8	6.63	95.2
7	25	66	7.5	0.45	5.0	6.0	95.0
8	50	40	11.85	1.05	7.9	8.86	92.1
9	72	54	3.8	0.77	2.53	20.26	97.47
10	25	70	7.4	1.73	4.93	23.37	95.07
11	32	58	8.46	1.28	5.6	15.13	94.4
12	53	80	9.8	0.6	6.53	6.12	93.47
13	20	66	7.4	0.15	4.93	2.03	95.07
14	26	74	4.0	0.21	2.66	5.25	97.34
15	56	60	5.48	0.7	3.65	12.79	96.35
16	43	50	5.4	1.1	3.6	20.37	96.4
17	28	74	4.25	1.58	2.83	37.17	97.17
18	59	65	4.9	0.6	3.27	12.24	96.73
19	46	80	4.3	1.03	2.87	23.88	97.13
20	34	66	52	0.57	3.46	10.96	96.54
21	53	60	5.05	0.37	3.36	7.32	96.64
AVERAGE	45.3	67.6	5.5	0.65	3.65	12.81	96.35

Fig. 1.

of Roe.[6] (7) Hematocrit determinations with a Wintrobe tube[5] also were made on each blood sample. (8) No food was allowed during the entire study period, but 250 c.c. of water were given at the beginning of the infusion and 250 c.c. at the end of the infusion. Therefore, the total fluid intake was 1,500 c.c. (9) Trained technicians (G. F. Lane and J. Holtzapple) administered all the infusions and remained with the patients throughout the period of injection. (10) Each infusion was kept at a steady rate of flow; the time required for the entire 1,000 c.c. was recorded. From this latter figure, the exact rate expressed as grams per kilogram per hour was calculated. (11) Oral temperature and blood pressure readings were recorded before and at the end of the test period. (12) Age of the subjects was noted also.

RESULTS

The average weight for the group was 68 kilograms (149 pounds) with a mean value of 66 kilograms (145 pounds). Weights ranged between 40 and 93 kilograms. Ages varied considerably between 20 and 72 years, but averaged 45 years. Thirteen of the twenty-one subjects were over the average age.

Administration rates were well maintained near the desired 1.5 Gm. per kilogram per hour. Actual rates of administration ranged from 1.3 to 1.7 Gm. per kilogram per hour and averaged 1.5 Gm. per kilogram per hour; the mean value was 1.6 Gm. per kilogram per hour. Eighty-nine minutes was the average time consumed for the infusion of 1,000 c.c. of 15 per cent invert sugar.

Total reducing substance recovered in urine samples collected for the entire four and one-half hour test period averaged 5.5 grams. The mean value was 5.1 grams. Review of the data for reducing substance excreted in each case showed a range between 0.5 and 11.8 Gm.; however, thirteen of the twenty-one subjects spilled less than the average value. The amount of sugar found in the urine expressed as per cent of the 150 Gm. injected was as follows: average per cent excretion of sugar infused 3.6, mean value 3.4 per cent, and a range of loss between 0.3 and 7.9 per cent.

Elderly patients are described as showing a low tolerance for glucose, and therefore a greater tendency to excrete the sugar injected.[7] Our analysis of the data in this report on parenteral invert sugar with this thought in mind does not indicate an analogous tendency. The subjects who lost more than 7 Gm. had an average age of 36, with a maximum age of 53, whereas those individuals who excreted less than 4.5 Gm. revealed an average age of 50 years with variations between 26 and 72.

Comparison of weights to per cent of sugar excreted tends to show a greater loss in the lighter subjects. Sixty-four kilograms is the average weight of patients who excreted more than 7 Gm., and 72 kilograms for the group spilling less than 4.5 grams.

The average of 0.6 Gm. of fructose is found in the urines collected for each subject. Hence, the fructose excreted in the urine represents 12.8 per cent of the total reducing substance found by the Benedict quantitative method. Since the invert sugar preparation is 50 per cent fructose and 50 per cent dextrose, and the rate of injection is 1.5 Gm. per kilogram per hour, the individual rates for each sugar component are 0.75 Gm. per kilogram. Inasmuch as the amount of fructose recovered is only 12.8 per cent of the total reducing substance in the urine, the implications are that fructose is more readily retained than is glucose, when they are injected simultaneously as invert sugar. Separate studies of fructose and dextrose and combinations of these two are being conducted to corroborate the foregoing hypothesis.

The per cent of invert sugar retained is the difference between that excreted and the amount injected. Therefore, 96.4 per cent of the 150 Gm. of invert sugar injected in eighty-nine minutes into the average adult is retained for utilization by the body.

Fig. 1 shows the ages, weights, grams of total reducing substance excreted in the urine, grams of fructose excreted in the urine, the per cent of infused sugar excreted, the per cent of total reducing substance recovered which appears as fructose, and the per cent of invert sugar retained for each subject studied.

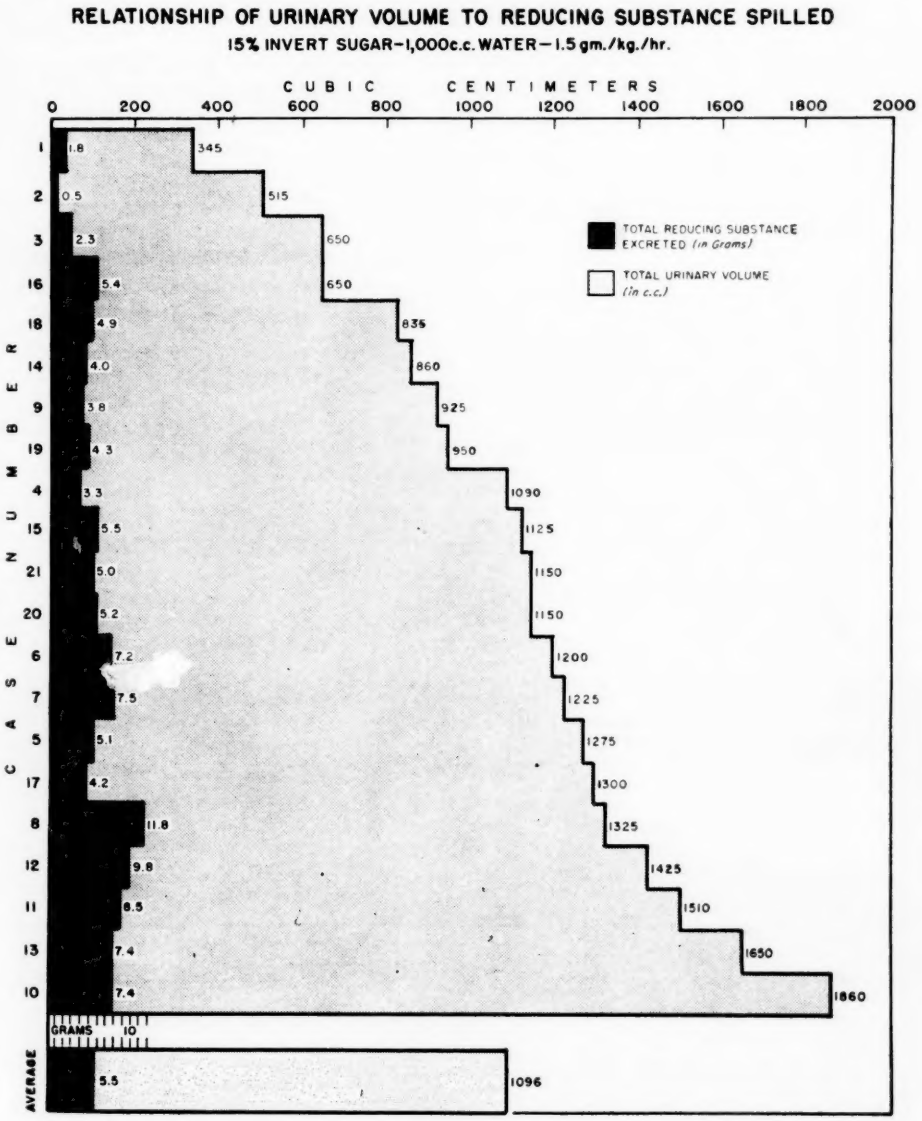

Fig. 2.

The urinary excretion for the subjects studied averaged 1,096 c.c. for each, with urinary volumes ranging between 345 c.c. and 1,860 c.c. Total fluid intake was 1,500 c.c. (1,000 c.c. by vein and 500 c.c. by mouth). Thus approximately 73 per cent of the fluid intake is excreted in the urine during the four and

one-half hour study period. This type of urinary response is considered to be characteristic of normal human beings.[8]

There is a direct relationship between degree of glycosuria and diuresis. In the group that excreted more than 1,000 c.c. of urine, an average of 6.7 Gm. of reducing substance was found; those who excreted more than 500 c.c., but less than 1,000 c.c., of urine spilled an average of 3.6 grams. One patient excreted only 345 c.c. of urine and the amount of urine sugar found was 1.8 grams. We feel that these figures indicate a rather close relationship between urine volume and grams of sugar spilled. Of course, some unpredictable variations far beyond this conclusion were also noted.

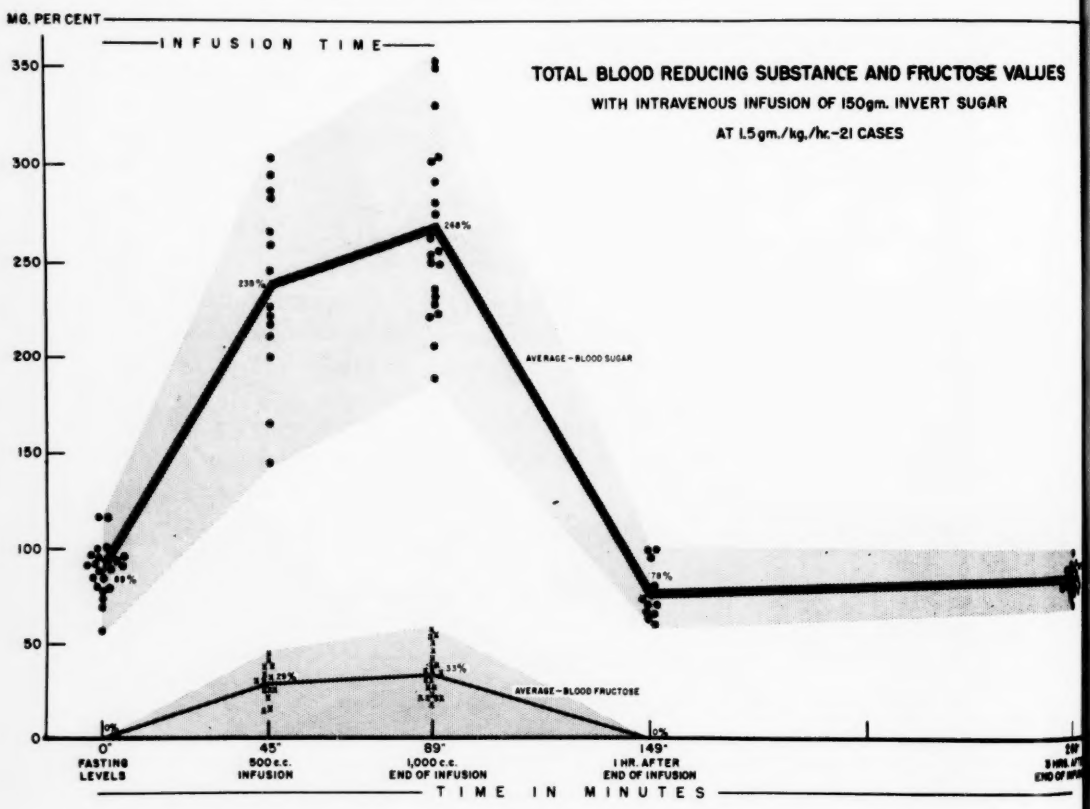
MG. PER CENT
INFUSION TIME
TOTAL BLOOD REDUCING SUBSTANCE AND FRUCTOSE VALUES
WITH INTRAVENOUS INFUSION OF 150gm. INVERT SUGAR
AT 1.5 gm./kg./hr.-21 CASES
AVERAGE-BLOOD SUGAR
AVERAGE-BLOOD FRUCTOSE
0" FASTING LEVELS
45" 500 c.c. INFUSION
89" 1,000 c.c. END OF INFUSION
149" 1 HR. AFTER END OF INFUSION
TIME IN MINUTES

Fig. 3.

Fig. 2 describes the total urinary volume for each case in order of increasing urinary output and the corresponding grams of total reducing sugar excreted in the urine.

Both the total reducing sugar and the fructose levels of the blood after the rapid injection of 150 Gm. of invert sugar are shown in Fig. 3. Blood sugar levels reach their maximum at the end of the infusion. A 300 per cent rise above preinfusion levels is noted. Within three hours after the end of the infusion, all blood values have returned to preinfusion levels. No definite hypoglycemic

reaction was found at this point. Fructose levels also reach their maximum height at the end of the infusion, with an average value of 33 mg. per cent. Fructose is not detectable in the three-hour postinfusion blood specimen. This rise in blood fructose represents only 18.4 per cent of the total rise in reducing sugar.

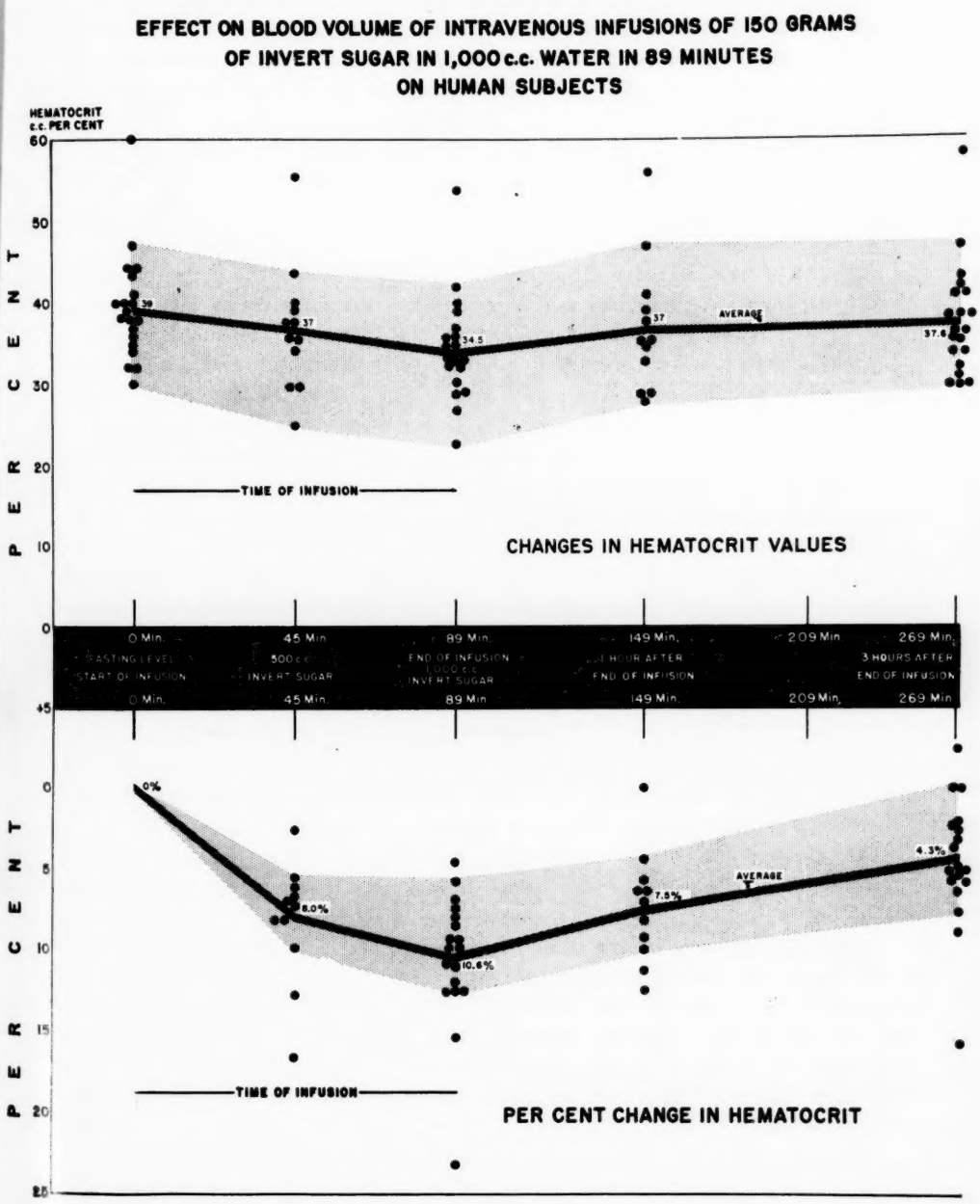

Fig. 4.

We previously mentioned that 13 per cent of the total reducing substance recovered in the urine was fructose. At the end of the infusion, the total blood reducing substance averages 268 mg. per cent, of which 33 mg. per cent is fructose. Fructose, therefore, represents 12.3 per cent of the total reducing substance in the blood at this time. The fact that 12.3 per cent of the total circulating reducing substance in the blood at the end of the infusion is fructose, and 13 per cent of the urine sugar is fructose, indicates that fructose is more readily removed from the blood and is retained more readily by the body and in greater quantity than is glucose, when both are given together as an invert sugar preparation.

Hematocrit values were determined during the study on each blood sample in an effort to estimate the degree and duration of hemodilution that develops when concentrated crystalloids are administered. A scattergraph of the hematocrit values obtained for each case along with the average values for the twenty-one subjects is represented in Fig. 4. Fig. 4 also describes the degree of hemodilution expressed as per cent change in hematocrit. An average 4.5 point drop in hematocrit reading is seen at the end of the infusion. This represents approximately a 10 per cent average hemodilution at this point, and a 10 per cent hemodilution represents between 450 and 500 c.c. increase in blood volume. Hematocrit values returned to preinfusion or near preinfusion levels at the end of the test period (three hours after completing the infusion). The average for all hematocrit levels at this point shows a 1.4 decrease in cubic centimeters per cent hematocrit from preinfusion levels. This represents a 4.3 per cent hemodilution. Such a minimal change is difficult to evaluate and is practically of no clinical significance. At no time during this study have we noticed any cardiac or pulmonary embarrassment from the administration of concentrated invert sugar solutions at a rate of 11 to 12 c.c. per minute.

Blood pressure and pulse rates before and after the infusions did not reveal any significant variations.

SUMMARY

The effect on the blood and on the urine of the administration of 150 Gm. of invert sugar in 1,000 c.c. of water as a single intravenous injection at a controlled rate of 1.5 Gm. per kilogram per hour is described.

An average loss of 3.6 per cent of 150 Gm. of sugar injected is noted. Therefore, 96.4 per cent of the sugar injected is retained by the body for utilization.

Only 12.3 per cent of the total circulating reducing substance in the blood at the end of the infusion is fructose, and only 12.8 per cent of the reducing sugar recovered in the urine is accounted for by fructose. These findings imply that fructose is more rapidly removed from the blood, is retained in greater quantities, and is more rapidly assimilated by the human body than is glucose when both sugars are given simultaneously at equal rates and in equal amounts as an invert sugar preparation.

Seventy-three per cent of the fluid intake is recovered in the urine during the four and one-half hour test period. This urinary response is considered to be normal for healthy adults.

A 10.6 per cent hemodilution occurs at the end of the infusion. This degree of hemodilution represents between 450 and 500 c.c. increase in blood volume. No clinical cardiac or pulmonary changes were noted which could be ascribed to the rapid administration of a concentrated solution of invert sugar.

Invert sugar can be given rapidly by vein in large quantities, and in moderate amounts of fluid, with the assurance of a minimal loss in the urine and a ready retention by the body for utilization.

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THE EFFECTS OF DIETARY DEPRIVATION OF POTASSIUM ON HEART GLYCOGEN AND ON BLOOD GLYCOLYSIS

SAM SEIFTER, PH.D., BROOKLYN, N. Y.

POTASSIUM, long considered to be the chief intracellular cation of many animal species, is known to play an important but ill-defined role in the metabolism of carbohydrate. Recently, Gardner and co-workers,[1] stimulated by the prominence currently attached to potassium in the origin of certain metabolic disturbances, made a study of the effects of dietary deficiency of this element on the carbohydrate metabolism of rats. They found that rats kept on a potassium-deficient diet for forty to sixty days showed abnormally high postprandial liver and muscle glycogen concentrations, but did not exhibit any abnormal response to an ingested dose of glucose. However, rats maintained on the same diet for ninety to one hundred twenty days showed almost no glycogen in the liver and skeletal muscle, and responded to orally ingested glucose with elevated tolerance curves. These authors concluded that their results indicated continued chronic alarm reaction ultimately leading to blocked glycogenesis due to the cumulative deficit of potassium.

The work reported in the present paper complements the studies of Gardner and his colleagues in that the effects of potassium deprivation on the glycogen contents of the specialized cardiac muscle, as well as the effects on blood glycolysis, were determined.

EXPERIMENTAL

Two series of rats were studied and the procedures applied to each are discussed separately.

Series I.—A group of male rats of the Wistar strain, each between 13 and 19 weeks of age and weighing between 260 and 290 grams, was maintained on a diet normal in all respects except for the content of potassium (Table I). The amount of this element offered the animals was at the low level of 0.1 mM per 100 Gm. of diet. Animals were sacrificed in subgroups as indicated in Table II after varying periods of time up to thirty-five days. Another group of rats was maintained on the potassium-deficient diet for thirty-five days and subsequently allowed to feed, for varying periods of time, on the same diet fortified with 11.5 mM of potassium per 100 Gm. of diet. All animals received adequate vitamin supplementation as described in a previous publication.[2] Only distilled water was allowed the animals for drinking purposes.

At the end of the experimental feeding period each animal was injected with the minimum dose of sodium pentobarbital necessary for anesthesia, its chest opened, and the rat exsanguinated by drawing blood from the heart with a syringe. The heart was then removed, uniformly trimmed, and dropped into weighed tubes containing 30 per cent KOH solution.

From the Department of Biochemistry, State University of New York, State University Medical Center at New York.

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After reweighing, the tubes were heated in boiling water for thirty minutes, and the glycogen content was determined by the method of Good, Kramer, and Somogyi[3] and by the indirect anthrone procedure.[4] No significant differences in glycogen contents of the same tissues were found as determined by the two methods. In this series of animals, it must be pointed out, the time elasping between administration of the anesthetic and excision of the heart was on the order of twenty minutes. In Series II, in contrast, the time elapsing between the administration of the anesthetic and submersion of the heart in KOH was approximately three minutes. As a consequence the control glycogen values were somewhat lower in Series I than those obtained in Series II.

TABLE I. COMPOSITION OF THE EXPERIMENTAL DIETS

DIET	CASEIN PER CENT	GLUCOSE PER CENT	SALT MIXTURE* PER CENT	CORN OIL PER CENT
Control	24.0	61.8	6.2	8.0
Low K	24.0	62.5	5.5	8.0

*Modified Orent-McCollum salt mixture (J. Biol. Chem. **140**: 337, 1941).
Control diet electrolytes in millimols per 100 Gm.: Na, 32; K, 11.5; Cl, 27.
Low K diet electrolytes in millimols per 100 Gm.: Na, 32; K, 0.1; Cl, 27.

TABLE II. AVERAGE HEART GLYCOGEN VALUES OF NORMAL, K-DEFICIENT, AND K-DEFICIENT RATS RESTORED TO A K-CONTAINING DIET (SERIES I)

DIET	NUMBER OF RATS	DAYS ON DIET	DAYS OF K SUPPLEMENTATION AFTER DEPLETION	HEART GLYCOGEN		
				GM. PER 100 GM. HEART	TOTAL MG. IN HEART	MG. PER 100 GM. BODY WEIGHT
Control	4	35	—	0.154	1.18	0.37
Low K	4	7	0	0.298	2.16	0.87
Low K	4	21	0	0.266	1.98	0.78
Low K	4	28	0	0.259	1.91	0.71
Low K	4	35	0	0.342	2.35	0.90
Low K	2	35	1	0.293	2.05	0.75
Low K	4	35	2	0.296	2.05	0.79
Low K	2	35	3	0.147	1.13	0.39
Low K	4	35	6	0.233	1.86	0.64
Low K	4	35	12	0.164	1.14	0.41

The red blood cells of the rats in Series I were separated according to the procedure described by Hegnauer[5] and analyzed for their contents of potassium by previously described methods.[6]

Series II.—Two groups of rats, similar with respect to sex, strain, age and weight to the animals in Series I, were placed on the potassium-deficient diet for forty-nine and seventy-seven days respectively. At the end of this time some of the animals in each group were sacrificed while others were continued for a few days before sacrifice on the same diet supplemented with 23 mM of potassium per 100 Gm. (see Table III for protocol). Suitable control animals were also studied.

The rats in Series II were sacrificed in a somewhat different manner from those in the first series. each animal received an intraperitoneal injection of 12 mg. of sodium pentobarbital, and one minute later was exsanguinated by cardiac puncture. The chest of the rat was then opened, and its heart excised, trimmed, and delivered into a tube containing hot KOH. Glycogen was then determined by the indirect anthrone procedure.[4]

The withdrawn blood was immediately defibrinated by the use of glass beads and was then transferred to a 20 ml. beaker. In this vessel, with its surface in contact with an air-water vapor phase, the blood was incubated in a Dubnoff metabolic shaker for three hours at 37.5° C. while being shaken at the rate of 96 times per minute. Samples were withdrawn

from the beaker initially and at one-hour intervals for analysis. Glucose was determined by the method of Somogyi[7] using the copper reagent of Nelson.[8] Lactic acid was determined by the method of Barker and Summerson,[9] and hemoglobin by that of van Bruggen.[10]

RESULTS

The control rats all gained weight during the experimental feeding period, while the potassium-deficient animals either gained a comparatively smaller amount or maintained their starting weights. At sacrifice the average weight of control animals was approximately 20 grams more than the average weight of experimental animals.

TABLE III. AVERAGE HEART GLYCOGEN VALUES OF NORMAL, K-DEFICIENT, AND K-DEFICIENT RATS RESTORED TO A K-CONTAINING DIET (SERIES II)

DIET	NUMBER OF RATS	DAYS ON DIET	DAYS OF K SUPPLEMENTATION AFTER DEPLETION	HEART GLYCOGEN		
				GM. PER 100 GM. HEART	TOTAL MG. IN HEART	MG. PER 100 GM. BODY WEIGHT
Control	4	50	—	0.207	1.67	0.51
Low K	3	49	0	0.287	2.09	0.73
Low K	3	49	2	0.253	1.90	0.62
Control	4	78	—	0.235	2.06	0.59
Low K	3	77	0	0.429	2.99	0.88
Low K	3	75	4	0.250	1.92	0.55

The data presented in Table II demonstrate that the rats in Series I maintained on a potassium-deficient diet showed a rapid and absolute increase of the glycogen contents of their hearts. In the case of the animals deprived of dietary potassium for thirty-five days, the average heart glycogen content was double that of the average control value although the size of the heart of an average potassium-deficient animal was considerably smaller than that of a control animal. After a period of twelve days of realimentation with potassium, the average heart glycogen contents of rats previously deprived of dietary potassium for thirty-five days showed a strong tendency to return to the average control value.

Analysis of the red blood cells of the rats in this series for their content of potassium revealed no significant deviation from the control value even after thirty-five days of dietary deficiency. This result is in contrast to that obtained with forty-one to forty-nine day potassium-deficient rats by Hegnauer,[5] who found a 10 per cent decrease in erythrocyte potassium content.

Table III shows the results obtained with the rats of Series II with respect to heart glycogen content. It is apparent that rats deprived of dietary potassium for forty-nine and seventy-seven days respectively showed an increase in heart glycogen content expressed in terms of concentration or in terms of absolute amount. Dietary administration of potassium to animals previously rendered deficient for similar periods of time, however, tended to cause a rapid restoration of heart glycogen to control concentrations and amounts.

Table IV shows that in both control and potassium-deprived rats in Series II the glycolytic rate of the blood as determined by the in vitro disappearance

of glucose was the same. This was true regardless of the method of expression of glycolytic rate, inasmuch as no significant deviations were found between potassium-deficient and control animals with respect to hemoglobin concentrations and red blood cell counts. Table IV also shows that rats deprived of potassium for seventy-seven days exhibited no significant difference from control rats in the capacity of their blood to metabolize glucose to lactic acid. It should be stated that the initial blood glucose and lactic acid values for both groups of rats were in the same range, and closely approximated normal values.

DISCUSSION

Gardner and co-workers[1] found that the liver and skeletal muscle glycogen of rats deprived of dietary potassium for forty to sixty days was more than doubled in concentration over control values. The present study shows that rats deprived of potassium for thirty-five days exhibited a doubling of the heart glycogen content as compared with suitable controls. While no figures are available for the potassium content of the heart under these conditions, it may be of some significance that a previous study from this laboratory[2] showed the plasma potassium level to be diminished by 50 per cent from the control value in rats maintained on the same low potassium diet for thirty-five days.

In the series of rats fed a low potassium diet for ninety to one hundred twenty days, as described in the paper of Gardner and associates, liver and skeletal muscle glycogen contents dropped to an extremely low level, whereas in the present series of animals the heart glycogen content continued to be double the control value after seventy-seven days of dietary potassium deprivation. Whether further potassium restriction would cause a loss of cardiac muscle glycogen cannot at present be answered, but the failure of the heart to relinquish its glycogen readily is not inconsistent with the generally unique behavior of glycogen in this organ.

TABLE IV. AVERAGE BLOOD GLYCOLYTIC RATES OF NORMAL, K-DEFICIENT, AND K-DEFICIENT RATS RESTORED TO A K-CONTAINING DIET (SERIES II)

DIET	NUMBER OF RATS	DAYS ON DIET	DAYS OF K RESUPPLEMENTATION	HB. (GM. %)	R.B.C. (MILL. PER C.MM.)	GLYCOLYTIC RATE (MG. GLUCOSE PER HR.) PER 100 ML.	PER GM. HB.	$\times 10^5$ PER MILL. R.B.C.	% CONVERTED GLUCOSE TO LACTIC ACID
Control	4	50	—	16.8	—	50.7	3.04	—	—
Low K	3	49	0	16.2	—	48.5	3.03	—	—
Low K	3	49	2	16.9	—	46.5	2.57	—	—
Control	4	78	—	14.4	7.73	49.7	3.49	6.42	55.1
Low K	3	77	0	14.2	8.59	55.0	3.87	6.49	45.0
Low K	3	75	4	14.2	7.50	48.0	0.35	6.88	61.2

The outstanding feature of the results obtained is the occurrence of an *absolute* increase in the amount of glycogen in the *smaller* hearts of the potassium-deficient animals. This can be explained by one of several assumptions: (a) in the absence of an adequate dietary intake of potassium, synthesis of glycogen

in the heart can proceed at a normal rate whereas glycogenolysis is decreased, or (b) glycogenesis (or gluconeogenesis) is accelerated although glycogenolysis is normal, or (c) both glycogenesis and glycogenolysis are inhibited, but glycogenolysis relatively more so. Since potassium is thought to play a role in both glycogenolysis and glycogenesis, it is difficult to speculate, in the absence of more information, on the metabolic processes of the heart during potassium deprivation.

It is interesting to note that during starvation[11] and in diabetes mellitus[12] the glycogen content of the heart may increase markedly, and some workers have ascribed this phenomenon in the latter case to the mass action effect on glycogenesis due to increased blood glucose levels. However, in the present case of potassium deprivation, the increase of cardiac glycogen occurs in the absence of abnormal blood glucose levels. Since insulin restores the cardiac glycogen contents of diabetic animals to normal levels, it would be of some interest to determine the effects of insulin on the cardiac, liver, and muscle glycogen of potassium-deficient rats.

Finally, in view of the fact that rats made potassium deficient for seventy-seven days showed no abnormality in blood glycolytic rate and no increase of this rate after the administration of potassium, it would appear that the amount of potassium in the rat erythrocyte under conditions of dietary deprivation is sufficient for the normal glycolytic activities of the cell, and any potassium which enters the red cells from the plasma following realimentation does so as osmotic equilibrium is being re-established, rather than as a response to an increase of the carbohydrate metabolism of the cell.

SUMMARY

Rats deprived of dietary potassium for periods of time varying from seven to seventy-seven days show a comparatively rapid absolute increase in the glycogen contents of their hearts as compared with suitable controls. The cardiac glycogen level of such potassium-starved rats, which is of the order of twice the normal value, can rapidly be restored to the control level by realimentation with potassium for several days.

Rats maintained on a potassium-deficient diet for forty-nine and seventy-seven days respectively exhibited no abnormal blood glucose and lactic acid levels, and no over-all qualitative or quantitative differences in blood glycolysis.

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OBSERVATIONS ON THE ABSORPTION, UTILIZATION, AND EXCRETION OF VITAMIN B_{12}

C. Lockard Conley, M.D., Julius R. Krevans, M.D., Bacon F. Chow, Ph.D., Charles Barrows, B.S., and Calvin A. Lang, A.B.
Baltimore, Md.

THE discovery of vitamin B_{12} and the study of its role in the therapy of pernicious anemia have led to new concepts of the pathologic physiology of this disease. Present evidence suggests that in pernicious anemia a vitamin B_{12} deficiency state exists. The vitamin B_{12} deficiency in this condition is apparently attributable to impaired absorption of the vitamin from the intestine. Normal absorption of B_{12} appears to be dependent upon the presence in the gastrointestinal tract of a substance secreted by the stomach. Deficiency of this "intrinsic factor" seems to be the cause of the absorption defect in pernicious anemia.

In preliminary studies an attempt was made to measure the absorption of vitamin B_{12} by determination of the rate of its urinary excretion in human subjects.[1] It was found that the vitamin B_{12} activity of the urine, as measured by a microbiological assay, was negligible in normal individuals and in patients with pernicious anemia. Following the parenteral administration of 100 micrograms of vitamin B_{12}, there was a prompf increase in the B_{12} activity of the urine, accounting for more than one-half of the injected vitamin within eight hours after injection. No difference was detected between the urinary response of normal individuals and that of patients with previously untreated pernicious anemia. However, when vitamin B_{12} was administered orally in single amounts as large as 500 micrograms, there was no measurable increase in the B_{12} activity of the urine even in normal individuals. This observation suggests that vitamin B_{12} may be poorly absorbed from the normal gastrointestinal tract.

In the experiments to be described, further observations have been made on normal individuals and on patients with pernicious anemia. Varying amounts of vitamin B_{12} have been administered orally, intramuscularly, and intravenously, and the rate of urinary excretion has been estimated. In some instances the concentration of B_{12} in plasma was determined. On the basis of these studies it was not possible to demonstrate a difference between normal individuals and patients with untreated pernicious anemia.

From the Clinical Microscopy Division, Department of Medicine, The Johns Hopkins University and Hospital, and the Department of Biochemistry, School of Hygiene and Public Health, The Johns Hopkins University.

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CLINICAL AND EXPERIMENTAL STUDIES

I. The Minimum Amount of Parenteral Vitamin B_{12} Necessary to Overcome the Manifestations of Pernicious Anemia.—It has been observed that minute quantities of vitamin B_{12} can correct the hematologic abnormalities of pernicious anemia in relapse.[2] Amounts varying between 25 and 100 micrograms administered in a single parenteral injection have been observed to produce a complete hematologic remission.[3, 4, 5] Reliable information is not available concerning the minimum quantity of vitamin B_{12} necessary for maximal improvement of the neurologic manifestations of the disease. Bortz has suggested that relatively massive doses of B_{12} are required for optimal effect on the nervous system.[6]

The following case reports indicate that both the hematologic and neurologic manifestations may respond to very small amounts of vitamin B_{12}.*

Patient W. B. (JHH No. 159322), a 65-year-old Negro laborer, complained of weakness, loss of weight, and dyspnea of six months' duration. He had noted difficulty in walking and numbness and tingling in the legs for two weeks, but no soreness of the tongue. Physical examination showed an elderly Negro man who looked chronically ill. The sclerae were slightly icteric, the conjunctivae pale. Papillary atrophy of the tongue was seen. Neurologic examination showed position sense to be absent in the toes and vibratory sense diminished in the lower extremities. The laboratory studies on admission gave the following results: red blood cells 1,540,000, hemoglobin 6.3 Gm., hematocrit 18.8, MCV 122, MCH 41, MCHC 34, white blood cells 3,300, platelets 156,000, icteric index 7.5. Aspirated sternal marrow contained many megaloblasts. No free acid was present in the gastric secretion after injection of histamine. He was given a single intramuscular injection of 12.5 micrograms of vitamin B_{12}. Reticulocytes reached a peak of 25 per cent five days after the injection. His hematocrit rose steadily. One month after this injection his hematocrit seemed to stabilize at 27 per cent. Therefore he was given another injection of 12.5 micrograms of vitamin B_{12} intramuscularly. His hematocrit rose to a normal level, and he showed marked subjective improvement. Vibratory and position sense returned to normal. No further therapy was given until three months after the first B_{12} injection. At this time the patient continued to feel well, and there was no evidence of clinical or hematologic relapse.

Patient F. W. (JHH No. 484433), a 56-year-old white housewife, complained of numbness of the arms and legs of two months' duration. One brother had died of pernicious anemia, and a sister of carcinoma of the stomach. Six years before admission the patient had noted weakness, lethargy, and sore tongue. She was told she was anemic, and administration of "vitamin C" was followed by marked improvement. Ten months before admission symptoms recurred and she developed diarrhea and sore tongue. Five months before admission she was found to be anemic and was treated by her physician with folic acid which promptly relieved her symptoms. Two months prior to admission she noted numbness in the arms and legs. This progressed rapidly until the time of admission when she complained of numbness of the lower extremities and of the upper extremities from the elbows to the fingertips. Her gait was unsteady, and she could not walk without assistance. She had difficulty in controlling her bladder. On physical examination atrophy of the papillae of the tongue was noted. There was glove and stocking hypoesthesia. Vibration sense was absent from the fingertips to the olecranon processes and from the toes to the anterior superior iliac spines. Position sense was lost in all extremities, and the Romberg sign was markedly positive. The laboratory studies on admission gave the following results: red blood cells 4,100,000, hemoglobin 14 Gm., hematocrit 43, MCV 104, MCH 34, MCHC 32, white blood cells 3,550. Gastric secretion contained no free acid after the injection of

*In these two studies vitamin B_{12} concentrate (Rubramin, Squibb) was used. In the remainder of the studies crystalline vitamin B_{12} (Cobione, Merck) was employed.

histamine. Sternal marrow smears showed large metamyelocytes, but no megaloblasts were seen. She was given a single intramuscular injection of 25 micrograms of vitamin B_{12}. Forty-eight hours later the patient noted subjective improvement in her gait and diminution of paresthesias. Two weeks after the injection vibration sense had returned to the hands and position sense was intact except in the toes. Therapy was withheld until six weeks after the injection. At this time her gait was entirely normal and position sense was intact. There was some tingling in the toes, and the vibration sense was slightly impaired in the legs.

These cases demonstrate that striking hematologic and neurologic improvement can follow the parenteral administration of as little as 25 micrograms of vitamin B_{12}. Although no further therapy was given for three months in one of these cases, there was no evidence of relapse.

II. The Minimum Amount of Oral Vitamin B_{12} Necessary to Overcome the Manifestations of Pernicious Anemia.—Vitamin B_{12}, when administered orally, is therapeutically effective in pernicious anemia.[7] The effectiveness of liver preparations in the treatment of pernicious anemia has been shown to be in direct relation to their vitamin B_{12} activity.[8] The dietary regimen prescribed by Minot and Murphy[9] was effective, presumably because of the high B_{12} content. The effective oral dose of crystalline vitamin B_{12} appears to be of the order of 40 to 150 times the minimal parenteral dose.[7, 10] The addition of normal gastric juice to orally administered vitamin B_{12} appreciably decreases the amount of B_{12} necessary for therapeutic effect.[11, 12] However, if the amount of B_{12} given orally is sufficiently large, gastric juice or other adjuvant factors may not be required.

Patients with pernicious anemia in relapse were given a single oral dose of vitamin B_{12}. Observations were made to determine the amount of B_{12} required to produce complete hematologic remission.

Patient C. J. (JHH No. 421071), a 50-year-old Negro woman, was admitted to the Johns Hopkins Hospital in July, 1947, because of severe anemia. A diagnosis of pernicious anemia was established, and she was treated with liver extract. Following her discharge from the hospital she failed to continue her prescribed therapy. She was seen again in July, 1950, when she complained of diarrhea, sore tongue, fatigue, and progressive weakness of the left leg. On physical examination she had a smooth tongue and a palpable spleen. Neurologic examination was normal except for hyperactive reflexes at the left ankle and knee. On admission the following laboratory data were obtained: red blood cells 1,280,000, hemoglobin 4.8 Gm., hematocrit 15.3, MCV 119, MCH 37, MCHC 31, white blood cells 6,800, platelets 22,000. Histamine refractory achlorhydria was demonstrated. Examination of marrow smears showed abnormalities typical of pernicious anemia. She received 5,000 micrograms of vitamin B_{12} as a single oral dose. No gastric juice or other adjuvant factor was added. There was rapid clinical improvement. The glossitis and the neurologic abnormalities disappeared. The hematologic response following the oral administration of this single dose of vitamin B_{12} was more rapid than the response previously observed following intensive parenteral liver therapy (Figs. 1 and 2).

Patient L. H. (JHH No. 542423), a 67-year-old Negro man, complained of weakness of two years' duration and progressive dyspnea on effort. Over the past few months there had been emotional lability, memory loss, and paranoid trends. The patient also noted paresthesias of the fingers and toes and some difficulty in walking. On examination the patient was cooperative but poorly oriented. Conjunctivae and mucous membranes were pale. The tongue showed papillary atrophy. Neurologic examination disclosed a markedly positive Romberg sign and loss of vibratory sense in both feet. Laboratory tests on admission gave the following results: red blood cells 1,100,000, hemoglobin 4.8 Gm., hematocrit 15, MCV

136, MCH 43, MCHC 32, white blood cells 1,800, platelets 26,000. Gastric secretion contained no free acid after histamine. Sternal marrow smears showed megaloblasts. The patient was given 2,000 micrograms of vitamin B_{12} orally in a single dose. Within three weeks his tongue was normal, the paresthesias had decreased, and there was striking mental improvement. There was a prompt hematologic response (Fig. 2). Three weeks following the administration of B_{12} the hematocrit appeared to reach a plateau at 31 per cent. At this time he was given parenteral B_{12} therapy, following which a slight secondary reticulocyte response was observed.

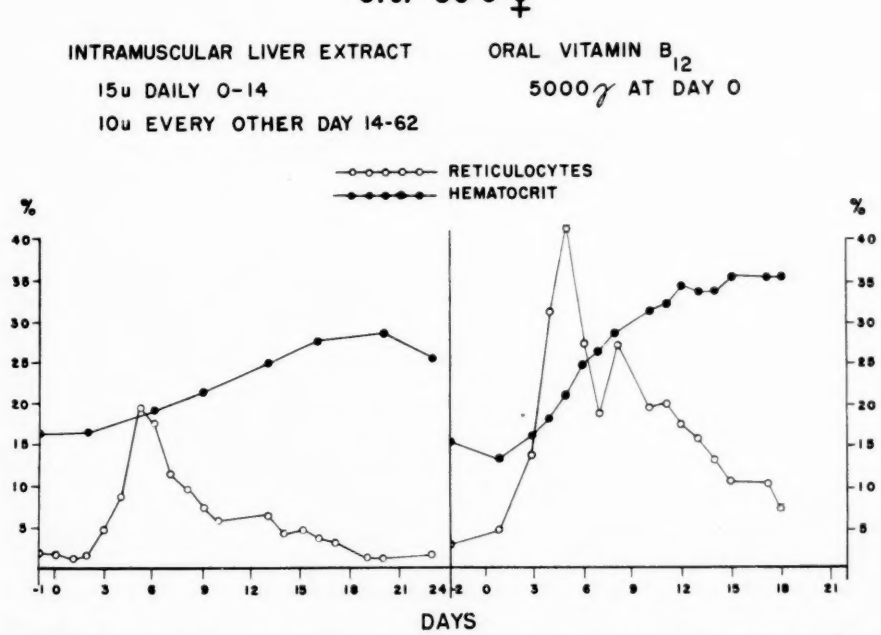

Fig. 1.—The hematologic response to parenteral liver extract therapy in a patient with pernicious anemia is compared to the response obtained in the same patient following oral administration of a single dose of vitamin B_{12}. The initial relapse occurred in 1947, and the second in 1950. Blood values prior to therapy on these two occasions were similar.

Patient F. T. (JHH No. 526576), a 58-year-old Negro woman, complained of weakness, loss of appetite, diarrhea, abdominal pain, numbness and tingling in the hands and feet, and burning of the tongue for six months. Physical examination on admission showed marked pallor of the mucous membranes. Several flame shaped hemorrhages were seen in the retinae. The papillae of the tongue were atrophic. No neurologic abnormalities were detected. Initial laboratory data were: red blood cells 1,500,000, hemoglobin 4.8 Gm., hematocrit 16, MCV 107, MCH 32, MCHC 30, white blood cells 1,150, platelets 46,000. Aspirated marrow showed megaloblastic hyperplasia. Gastric analysis revealed a histamine-fast achlorhydria. The patient was given 1,000 micrograms of vitamin B_{12} orally as a single dose. This was followed by rapid clinical improvement. There was a prompt hematologic response (Fig. 2). Sixteen days after the administration of B_{12} the hematocrit appeared to level off at 26 per cent. Parenteral administration of vitamin B_{12} was followed by a secondary reticulocyte response.

These observations demonstrate that 5,000 micrograms of vitamin B_{12}, when administered alone in a single oral dose, was effective in inducing complete hematologic remission in a patient with pernicious anemia. Single oral doses of

1,000 and 2,000 micrograms were followed by striking hematologic changes, but were not adequate to restore the blood values to normal. Since approximately 25 micrograms of B_{12} given parenterally can produce complete remission, it seems probable that the patient who received 5,000 micrograms orally absorbed 25 micrograms or more of the ingested vitamin B_{12}.

III. The Plasma Concentration and Urinary Excretion of Vitamin B_{12} Following Its Intravenous Administration.—Two normal young adults and two patients with pernicious anemia in remission were given 1,000 micrograms of vitamin B_{12} intravenously. Blood and urine samples were collected at intervals. Two other normal subjects received 25 micrograms of vitamin B_{12} by intravenous injection.

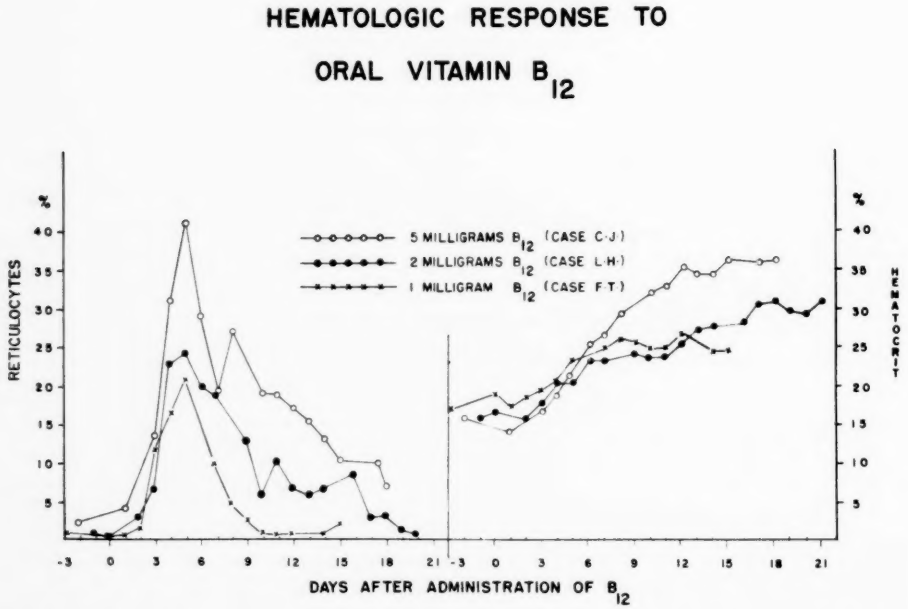

Fig. 2.—Hematologic changes in three patients with pernicious anemia following a single oral dose of vitamin B_{12}.

Urine and blood samples were collected from the subjects to be studied. One thousand micrograms of vitamin B_{12} dissolved in 1 ml. of water were injected into an antecubital vein. The syringe was flushed by withdrawing blood and reinjecting several times. No untoward reactions were noted in any subject following the injection. Subsequent blood samples were drawn from the other arm at various intervals for twenty-four hours. All urine was collected over the same period of time. The blood was oxalated by introducing 10 ml. of blood into a bottle containing 15 mg. of potassium oxalate. Within one hour the plasma was separated by centrifugation. The oxalated plasma was frozen immediately and stored in the frozen state until analyses were performed. Urine specimens were collected in acid cleaned bottles containing 5 ml. of toluol. At the end of each collection period the volume of the samples was measured and aliquots frozen for analysis. The B_{12} activity of the urine was estimated by a microbiological method.[13] In order to ascertain the validity of the microbiological method, vitamin B_{12} was added to normal urine in concentrations varying from 0.04 to 1.0 millimicrograms per milliliter. Recovery was possible within

∓ 20 per cent. Multiple determinations on the same specimen gave results that agreed within 20 per cent. One part of plasma was dialysed against 9 parts of 0.85 per cent sodium chloride solution and the dialysate assayed for B_{12} microbiologically. Recovery experiments demonstrated that vitamin B_{12} was equally distributed between the plasma and the saline dialysate.

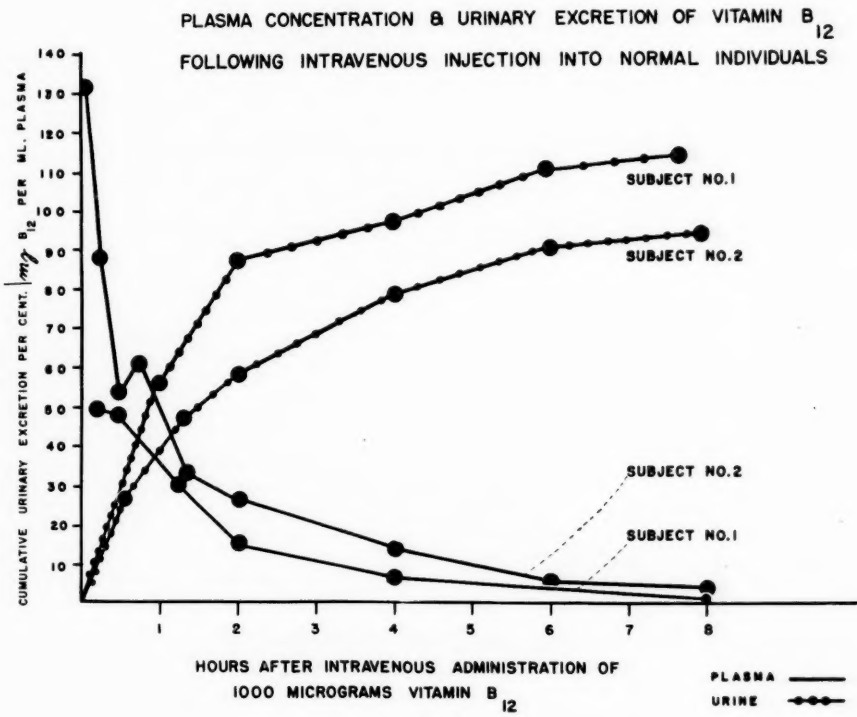

Fig. 3.

Urine was collected from the subjects who received 25 micrograms of vitamin B_{12}, but blood examinations were not performed. The results of this study are shown in Figs. 3 and 4. Normal plasma and plasma of the patients with pernicious anemia in remission showed vitamin B_{12} activity equivalent to less than 3.5 millimicrograms per milliliter. Following the intravenous injection of 1,000 micrograms of vitamin B_{12}, the B_{12} activity of plasma greatly increased. Six hours after the injection, increased B_{12} activity was no longer demonstrable in the plasma. The urine obtained from each of the subjects prior to injection of B_{12} showed no measurable B_{12} activity. Following the injection approximately 100 per cent of the injected B_{12} could be accounted for in the urine within eight hours. The high B_{12} activity of the urine suggests that vitamin B_{12} excretion is rapid and approximately quantitative following parenteral administration of these large amounts. There was no detectable difference between the normal subjects and the patients with pernicious anemia in this study.

Following the intravenous injection of 25 micrograms of vitamin B_{12}, the urine excreted in the first four hours showed increased B_{12} activity. In these

subjects the activity of the urine accounted for less than 15 per cent of the injected material.

IV. The Urinary Excretion of Vitamin B_{12} Following Its Intramuscular Injection.—In the preliminary study previously reported, the B_{12} activity of urine from normal individuals and patients with pernicious anemia was measured following intramuscular injection of the vitamin.[1] These observations have been expanded to include the urinary response to widely varying amounts of parenterally administered vitamin B_{12}.

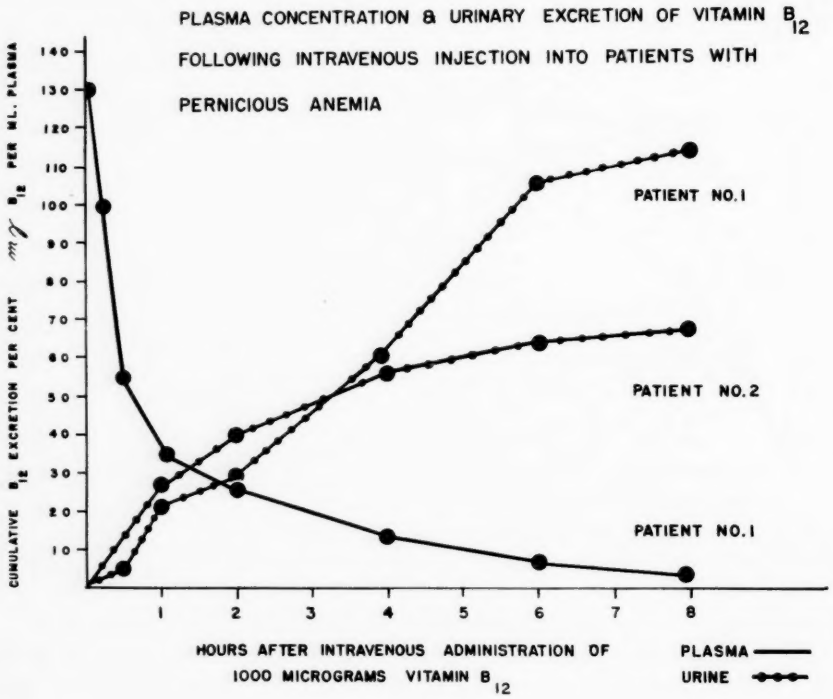

Fig. 4.

Eleven normal subjects and five patients with untreated and treated pernicious anemia were used in this study. The subjects were maintained on a vitamin B_{12} deficient diet, which was started at least twenty-four hours before the beginning of the experiment. Vitamin B_{12} was given intramuscularly as follows: 15 micrograms, 1 subject; 25 micrograms, 3 subjects; 100 micrograms, 5 subjects; and 500 micrograms, 7 subjects. Urine was collected for twenty-four hours prior to injection and for forty-eight hours thereafter. Urine samples were collected in acid cleaned bottles containing 5 ml. of toluol. The urine volume was measured and aliquots were frozen and stored for assay.

The results of this study are presented in Fig. 5. Following the intramuscular injection of 25 micrograms or less of vitamin B_{12}, no significant increase in the B_{12} activity of the urine was detected. The vitamin B_{12} activity of the urine excreted in the first eight hours following the injection of 100 micrograms was greatly increased. In five subjects this activity accounted for 19 to 49 per cent of

the injected vitamin. When 500 micrograms were administered to seven subjects, the B_{12} activity of the urine excreted in the eight hours following injection ranged from 86 to 125 per cent of the injected vitamin. The indicates an excretion of approximately 100 per cent, the variation falling within the error of the method. No difference was demonstrated between the apparent rates of excretion of normal subjects and of patients with pernicious anemia.

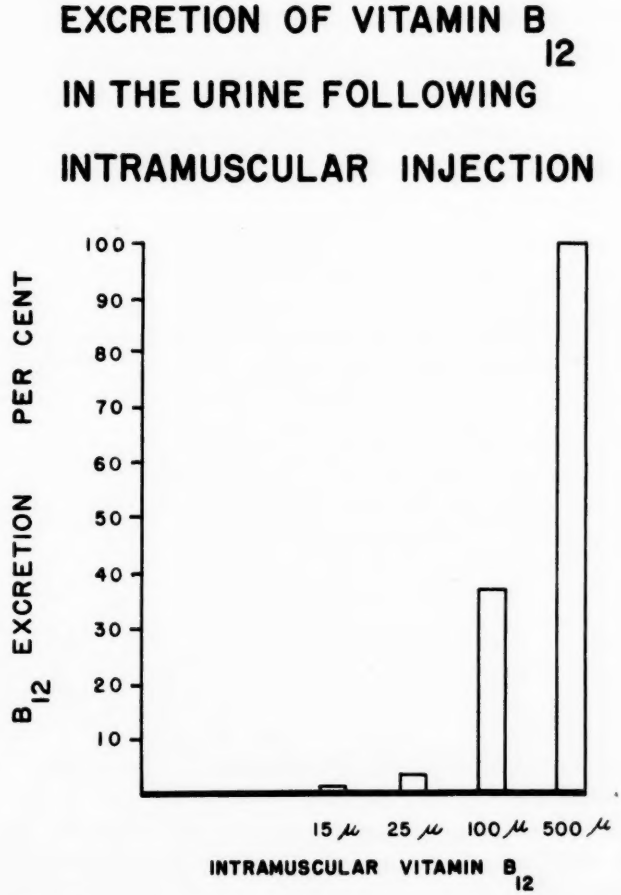

Fig. 5.—The B_{12} content of urine collected for forty-eight hours after intramuscular injection of various amounts of vitamin B_{12} is expressed as per cent of the vitamin B_{12} injected. The data are based on sixteen experiments, and the values recorded are the averages of the results of several experiments at each dosage level.

V. The B_{12} Activity of Urine Following Oral Administration of the Vitamin.—Previous observations had indicated that there was no measurable increase in the B_{12} activity of urine following the oral administration to normal subjects of vitamin B_{12} in amounts as large as 500 micrograms.[1] Additional experiments were performed in which normal subjects and patients with pernicious anemia were given vitamin B_{12} orally in single amounts as large as 10,000 micrograms.

In several experiments folic acid in amounts ranging from 5 to 20 mg. was administered with the vitamin B_{12}. The urine was collected and assayed for B_{12} activity.

The subjects included normal individuals and patients with treated and untreated pernicious anemia. All subjects were placed on a diet containing no meat or fowl and including only one egg and one glass of milk daily. Urine was collected for twenty-four hours. Vitamin B_{12} in an amount of 1,000, 2,000, 5,000 or 10,000 micrograms was given with water before breakfast. No food was allowed for one hour following ingestion of the B_{12}. Urine specimens were collected, measured, and assayed as in Experiment IV.

TABLE I. URINARY EXCRETION OF VITAMIN B_{12} FOLLOWING ORAL ADMINISTRATION

VITAMIN B_{12} (μG)	SUBJECTS	EXCRETION
1,000	1 (pernicious anemia)	0*
2,000	3 (normal)	0*
	1 (pernicious anemia)	0*
5,000	4 (normal)	0*
	4 (pernicious anemia)	0*
10,000	1 (normal)	0*
	1 (pernicious anemia)	0*
500 + 20 mg. folic acid	3 (normal)	0*
5000 + 5 mg. folic acid	1 (pernicious anemia)	0*

*B_{12} activity in urine was less than 0.05 millimicrograms per milliliter.

The vitamin B_{12} activity of the urine collected for forty-eight hours following the oral administration of B_{12} to normal subjects and to patients with pernicious anemia is recorded. Four subjects received folic acid, as indicated, in addition to vitamin B_{12}.

The results are presented in Table I. In no instance was a measurable increase in B_{12} activity of urine observed. Nevertheless, in the three patients with pernicious anemia in relapse, a prompt hematologic response to the orally administered vitamin occurred. (See Section II.)

DISCUSSION

It is currently believed that pernicious anemia is a vitamin B_{12} deficiency state attributable to defective absorption of the vitamin from the gastrointestinal tract. When the vitamin is administered orally to patients with pernicious anemia in relapse, the hematologic response serves as an indirect measure of the absorption of B_{12}. However, no direct method is available for measuring the rate of absorption.

The rate of absorption of vitamin B_{12} from the intestinal tract of normal individuals is unknown. A method for measuring this rate would be particularly valuable for the following reasons:

1. This method would facilitate the study of factors which influence the absorption of vitamin B_{12}.
2. The method could provide a diagnostic test for pernicious anemia, particularly in those patients in whom the diagnosis has been made obscure by previous treatment.

An attempt was made to measure the absorption of vitamin B_{12} by determining the rate of its excretion in the urine.

The plasma and urine concentrations of vitamin B_{12} in normal individuals and in patients with pernicious anemia were too low for accurate measurement

by the method employed. In most instances the plasma concentration was less than 3.5 millimicrograms per milliliter and the urine concentration less than 0.05 millimicrograms per milliliter. Following parenteral administration of B_{12} in amounts exceeding 25 micrograms, there was a prompt increase in the B_{12} activity of the urine, suggesting that a considerable proportion of the injected vitamin was rapidly excreted in the urine.

When single amounts of vitamin B_{12} as large as 10,000 micrograms were administered orally to normal individuals and patients with pernicious anemia, no significant increase in the B_{12} activity of the urine was observed. These observations suggest that the concentration of B_{12} in body fluids following ingestion of the vitamin, at no time approached the levels produced by parenteral injection of 100 micrograms.

Clinical observations on patients with pernicious anemia in relapse showed that B_{12} was absorbed from the gastrointestinal tract when administered without gastric juice or other factors. However, it was necessary to administer approximately 200 times that amount, which, when given parenterally, would produce the same clinical effect.

The increase of B_{12} activity in urine following parenteral administration of vitamin B_{12} strongly suggests that when the B_{12} concentration in body fluids is high, there is rapid excretion in the urine. However, oral administration of exceedingly large amounts was not followed by measurable urinary excretion, even in normal individuals. When patients with pernicious anemia in relapse were fed vitamin B_{12} in these same large amounts, clinical response occurred, although no excretion in the urine could be demonstrated. It is apparent that the vitamin B_{12} activity of the urine cannot be used as a measure of absorption of small amounts of B_{12}. When 25 micrograms or less were given parenterally, there was no measurable urinary excretion.

Apparently the body needs only a small amount of B_{12} and normally can adequately absorb that amount from the intestinal tract. The amount of B_{12} which can be absorbed from the intestines of a normal individual appears to be limited. Possibly some mechanism regulates the amount absorbed depending on the needs of the body. The absorption of B_{12} in patients with pernicious anemia is not as strikingly different from that in normal individuals as had been anticipated.

SUMMARY

Parenteral administration of as little as 25 micrograms of vitamin B_{12} to patients with pernicious anemia in severe relapse may be followed by complete hematologic remission. When vitamin B_{12} is administered orally in a single dose without gastric juice, approximately 5,000 micrograms are required to produce the same effect.

Following parenteral administration of vitamin B_{12} in amounts larger than 25 micrograms, there was prompt urinary excretion of the vitamin. When 500 micrograms or more were injected, the urinary excretion of B_{12} was approximately quantitative. No difference was detected between the B_{12} excretion of normal individuals and of patients with pernicious anemia.

Oral administration of single amounts of vitamin B_{12} as large as 10,000 micrograms was not followed by a measurable increase in the B_{12} content of the urine of normal individuals or of patients with pernicious anemia.

ADDENDUM

Since this report was submitted, a patient with pernicious anemia in relapse had complete hematologic remission following the oral administration of a single dose of 3,000 μg of crystalline vitamin B_{12}.

The crystalline vitamin B_{12} used in most of these experiments was provided by Merck & Co., Inc., Rahway, N. J. Vitamin B_{12} concentrate used in two experiments was provided by E. R. Squibb & Sons, New York, N. Y.

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BIOCHEMICAL STUDIES WITH THE "CITROVORUM FACTOR"

H. P. Broquist, Ph.D., E. L. R. Stokstad, Ph.D., and T. H. Jukes, Ph.D.
Pearl River, N. Y.

IT WAS noted by Sauberlich and Baumann[1] that *Leuconostoc citrovorum* 8081 failed to grow on a synthetic medium unless supplied with an unidentified "citrovorum factor" (CF) which was present in a variety of natural materials and was later shown[2, 3] to be closely related to pteroylglutamic acid (PGA). Recently the synthesis from pteroylglutamic acid (PGA)[4, 5] and isolation of a crystalline substance[5] having the biological properties of CF was described. This crystalline substance, "leucovorin," was effective as a replacement for PGA in the nutrition of *Streptococcus faecalis, Lactobacillus casei,* and the chick.[6] In this report "leucovorin" refers to the synthetic crystalline free acid[5] while "CF" refers to the microbiological activity of crude materials for *Leuconostoc citrovorum.* All weights of leucovorin and CF are expressed in terms of the anhydrous free acid 0.15 mγ of which is equivalent to one "unit."[6] The term "pteroylglutamic acid" (PGA) refers to the specific chemical substance, while "folic acid activity" (FAA) refers to biological activity for *S. faecalis* R expressed in equivalence of PGA as a standard;[7] this biological property is shared by PGA and CF. CF concentrates[3] and leucovorin[6] were more effective than PGA in reversing antagonists of PGA for microorganisms and mice. These findings may indicate that CF is an important functional form of PGA in metabolism.

Sauberlich[8] reported that rats excreted CF in the urine in proportion to the amount of PGA in the diet, indicating a metabolic relation between PGA and CF. Similar results were subsequently noted with human subjects.[9] In the present investigation normal adult males were given PGA or CF under varying conditions, and the urinary excretion of CF was determined. Certain relationships between ascorbic acid and CF in human subjects and in guinea pigs were investigated.

EXPERIMENTAL

Normal adult men were used as subjects. It was found that on ordinary diets the urinary excretion of CF was low and large individual variations were not encountered; hence no attempt was made to control the diet. Urine samples were collected in brown bottles containing 0.1M pH 7.2 phosphate buffer to maintain neutrality and stored in the refrigerator under toluene.

The microbiological assay for CF with *Leuconostoc citrovorum* 8081 was similar to that of Sauberlich,[8] using leucovorin as a standard. Leucovorin was synthesized and supplied by Drs. Barbara Roth, M. E. Hultquist, and J. M. Smith, Jr., Calco Chemical Division, American Cyanamid Company. Folic acid

From the Lederle Laboratories Division, American Cyanamid Company.
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assays were carried out with *Streptococcus faecalis* R using crystalline PGA as a standard. The technique of culturing this organism was essentially that described by Broquist and Snell.[10]

The average CF content of normal urine was somewhat less than 0.001γ per millimeter (Table I). Jukes and associates[11] have reported that on ordinary diets the FAA of the urine is between 0.005 to 0.01γ per millimeter, so it would appears that all of the FAA is not in the form of CF.

The effect of a 50 mg. oral dose of PGA on the urinary excretion of CF was studied. Jukes and co-workers[11] found that following oral administration of 5 mg. of PGA at least 85 per cent of the subsequent increase in urinary PGA excretion could be recovered in the first six hours. In the present study a six-hour collection period was hence used. It was found (Table I) that following the administration of 50 mg. of PGA an average of 42γ of CF was excreted indicating that only about 0.1 per cent of the ingested PGA was converted to urinary CF. This observation indicates that the normal subject converts PGA to CF only at a slow rate or that CF is not readily excreted in the urine. The latter alternative does not appear to be probable in view of the results reported in Table IV.

TABLE I. EFFECT OF ADMINISTRATION OF PTEROYLGLUTAMIC ACID (PGA) AND ASCORBIC ACID ON URINARY EXCRETION OF CITROVORUM FACTOR (CF)

SUBJECT	CF CONTENT OF NORMAL URINE (γ/ML.)	TOTAL CF EXCRETED IN 6 HOURS FOLLOWING ORAL ADMINISTRATION OF 50 MG. PGA (γ)	TOTAL CF EXCRETED* IN 6 HOURS FOLLOWING ORAL ADMINISTRATION OF 50 MG. PGA + 1 GM. ASCORBIC ACID (γ)
1	0.0006	55	103
2	0.0004	36	116
3	0.0004	32	108
4	0.0006	52	121
5	0.0008	40	
6	0.0006	36	124
Average	0.0006	42	114

*This excretion experiment was performed several days later, after the CF content of the urine of all subjects on experiment had returned to normal (ca. 0.001γ CF/ml.)

Nichol and Welch recently reported[12] that CF was formed when rat liver slices are incubated in the presence of PGA. Under these conditions ascorbic acid appeared to augment significantly the formation of CF. These authors also noted briefly that the urinary excretion of CF following the ingestion of PGA was increased by ascorbic acid. Similar results were obtained in this investigation (Table II). The effect of a combined oral dose of PGA and ascorbic acid on the urinary excretion of CF was studied. The data of Table I shows that the simultaneous addition of ascorbic acid and PGA resulted in a threefold increase in the CF content of the urine. High levels of PGA will under certain conditions stimulate the growth of *L. citrovorum*.[1] Although this series of urine samples contained a high amount of PGA (ca. 100γ/ml.), the dilution employed for the CF assay would render PGA inactive at these levels. In separate experiments (Table IV) it was found that ascorbic acid did not increase the amount of CF recovered in the urine when ascorbic acid was taken orally with leucovorin.

Hence the possibility that ascorbic acid might function as a protective reducing agent was discarded. These data provide evidence for the involvement of ascorbic acid in the biosynthesis of CF in human subjects.

TABLE II. ENHANCEMENT OF CF SYNTHESIS BY RAT LIVER HOMOGENATE IN THE PRESENCE OF PGA AND ASCORBIC ACID*

ADDITION TO RAT LIVER HOMOGENATE (PER GM. OF LIVER)	CF CONTENT OF SUPERNATANT LIQUID FOLLOWING A TWO-HOUR INCUBATION PERIOD (PER GM. OF LIVER) (mγ)
1. None	45
2. 280γ PGA	69
3. 28 mg. ascorbic acid	390
4. 280γ PGA + 28 mg. ascorbic acid	890

*The technique employed in this experiment was exactly as described by Nichol and Welch[12] except that rat liver homogenate was used rather than rat liver slices. Weanling rats that had been on a PGA deficient diet for one month were used.

The results suggested that perhaps a major role of ascorbic acid in nutrition was to participate in the formation of CF and that administration of CF might alleviate a deficiency of ascorbic acid. An experiment was carried out with guinea pigs to determine if leucovorin could replace the requirement of the guinea pig for ascorbic acid. Guinea pigs were placed on a scorbutic diet of the following composition:

Ground whole oats	66%
Skim Milk powder heated at 100° C. for 24 hours	30
NaCl	1
Corn oil (Mazola) *plus* vitamins A, D, and E[13]	3
	100

After a depletion period of nine days, the animals were divided into three groups receiving respectively no supplement, ascorbic acid, and leucovorin. Nine animals were used per group, and the results are indicated in Table III. Animals receiving the basal diet with or without leucovorin rapidly lost weight and none survived the test period of twenty-five days; animals receiving ascorbic acid steadily gained weight and most of the animals survived. On the twenty-first day of the experiment urine samples were collected from the control group of guinea pigs and from the group receiving ascorbic acid. Over a fifteen-hour collection period each group excreted 22 mγ of CF. Hence with guinea pigs no evidence was found for a relationship between dietary ascorbic acid and the urinary excretion of CF.

The effect of mode of administration of leucovorin on the urinary excretion of CF and folic acid in human subjects was next studied and the results are summarized in Table IV. It was found that when leucovorin was given orally only 1.1 per cent of the vitamin was recovered in the urine and that the recovery was lowered by the simultaneous administration of ascorbic acid.

CF is rapidly destroyed at room temperature by dilute HCl, but its folic acid activity remains[3] indicating conversion of CF to another compound with folic acid activity. It seemed possible that on oral administration of CF the acidity of the gastric juice might carry out this change in vivo. However, the amount of FAA in the urine following oral dosage with leucovorin was only slightly higher than the CF content. Hence it appears that when CF is given

orally it is metabolized to some substance other than folic acid or is not absorbed. When CF was given by intravenous injection 22 per cent of the dose appeared in the urine, and the folic acid content was about the same as the CF content. These results may be compared with our finding[6] that leucovorin when incorporated in the diet or when fed by pipette to PGA deficient chicks gave a poor growth and hematopoietic response, but when given by injection the activity of leucovorin was comparable to that of PGA. The parenteral route for clinical administration of leucovorin is to be preferred in view of these findings.

TABLE III. INABILITY OF LEUCOVORIN TO REPLACE ASCORBIC ACID FOR THE GUINEA PIG

DAYS ON EXPERIMENT	WEIGHT IN GM. AND SURVIVORS (IN PARENTHESES) SUPPLEMENT (GIVEN BY SUBCUTANEOUS INJECTION 3 × WEEKLY)		
	NONE	2 MG. ASCORBIC ACID	300γ LEUCOVORIN
9	248(9)	248(9)	248(9)
11	243(9)	236(9)	232(9)
14	226(9)	249(9)	217(9)
16	206(9)	238(9)	250(7)
18	219(6)	257(7)*	213(6)
21	205(2)	262(7)	170(2)
23	185(2)	256(7)	145(1)
25	0	264(7)	0

*One animal was removed from this group as both hind legs were broken.

TABLE IV. EFFECT OF MODE AND ADMINISTRATION OF LEUCOVORIN ON THE URINARY EXCRETION OF CF AND FOLIC ACID (FA) IN ADULT MEN

SUBJECT	URINARY EXCRETION IN 6 HOURS FOLLOWING ORAL ADMINISTRATION OF 2.57 MG. LEUCOVORIN						URINARY EXCRETION IN 6 HOURS FOLLOWING INTRAVENOUS INJECTION OF 3.05 MG. LEUCOVORIN		
	WITHOUT ASCORBIC ACID			1 GM. ASCORBIC ACID					
	γ CF	PER CENT CF RECOVERED	γ FAA	γ CF	PER CENT CF RECOVERED	γ FAA	γ CF	PER CENT CF RECOVERED	γ FAA
1	28	1.1	50	4	0.16	18	682	22	630
2	39	1.5	63	13	0.5	29	679	22	970
3	32	1.2	66	12	0.5	20	725	24	620
4	31	1.2	51	19	0.7	30			
5	27	1.1	47	32	1.2	36	660	22	540
6	15	0.6	45				652	22	610
Average	29	1.1	54	16	0.6	27	680	22	674

DISCUSSION

Data presented in this paper indicate that on a normal diet all of the FAA in the urine is not in the form of CF. It is possible that an equilibrium exists between PGA and CF in the body. Further studies are required to determine whether CF is involved in the various biochemical reactions in which PGA is known to participate. There is evidence from microbiological studies that CF can function in the synthesis of thymidine[3, 14] and PGA is known to be concerned in the synthesis of purine bases and thymine.

Schoenbach has recently demonstrated[15] that PGA when administered in large doses to mice for a sufficient time period proceding administration of aminopterin is able to protect against the toxicity of the antagonist. In explanation of these results, it seems very likely that PGA is converted to CF, which is able successfully to reverse the antagonist. The present data bear upon the magnitude and rate of the conversion of PGA to CF in human beings.

In view of the involvement of vitamin C in the biosynthesis of CF, it is possible that the adrenals are concerned in this synthesis. Another circumstance linking an adrenal compound to FAA is the report[16] that dehydroisoandrosterone acetate, an androgen produced in the adrenals, appeared to replace the requirement of PGA for certain microorganisms.

The finding in this investigation that only about 0.1 per cent of an administered dose of PGA appeared as urinary CF is in good agreement with the conclusions drawn by Keresztesy and Silverman.[17]

SUMMARY

Normal adult males were given pteroylglutamic acid (PGA) or leucovorin (a synthetic substance having the activity of citrovorum factor [CF]) under varying conditions and the urinary excretion of CF was measured by microbiological assay.

The average CF content of normal urine was somewhat less than 0.001γ/ml. As judged by six-hour urinary excretion, 0.1 per cent of an orally administered dose of 50 mg. of PGA was converted to CF. About a three-fold higher level of CF was excreted if 1 Gm. ascorbic acid was taken simultaneously with 50 mg. PGA.

When leucovorin was taken orally only 1.1 per cent of the CF activity of the dose was recovered in the urine. This recovery was not increased by the simultaneous ingestion of ascorbic acid. When leucovorin was taken by injection 22 per cent of the activity was recovered in the urine.

When liver homogenate from PGA deficient rats was incubated in the presence of PGA a marked increase in the amount of CF formed was noted on the addition of ascorbic acid. However, leucovorin failed to alleviate a dietary deficiency of ascorbic acid in guinea pigs.

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THE EFFECT OF AUREOMYCIN AND PENICILLIN ON EXPERIMENTAL ACTINOMYCOSIS INFECTIONS IN MICE

ROBERT S. GEISTER, M.S., AND ESTHER MEYER, PH.D.
CHICAGO, ILL.

IN 1943 Keefer and associates[1] gave the first indication that penicillin may be of some aid in the treatment of this disease in man. Considerable literature has accumulated since this initial report concerning the effect of antibiotic agents on *Actinomyces bovis*. Reports indicate variable or unsuccessful results in vitro and in treatment. Despite a variation in strain susceptibility, penicillin has been the most effective agent for the treatment of actinomycosis.[2-6]

The primary purpose of this study was to investigate the in vivo activity of aureomycin and penicillin in actinomycosis infections of mice caused by the organism *Actinomyces bovis*. A secondary objective, which evolved during the experimental procedures, was to develop a method for grading the effect of these agents on the dissemination and progression of the disease process. No reports have appeared in the literature covering these subjects.

In control groups, mice were infected with *Actinomyces bovis*, and the progression of the disease was evaluated. Other groups of mice were infected with the same organism and treated both prophylactically and therapeutically with aureomycin and penicillin. A method for evaluating the relative therapeutic efficiency of these agents against this disease was developed.

It is well known that there are some marked discrepancies between in vitro susceptibility tests and the in vivo activity of a drug against an organism. There are also some discrepancies between animal therapeutic tests and results of clinical trials in human patients. Nevertheless an experimental in vivo method is highly desirable.[7] A survey of the literature indicates the lack of an in vivo method suitable for testing the efficacy of either chemotherapeutic or antibiotic agents against this organism. Recently, Meyer and Verges,[8] demonstrated that a high percentage of young albino mice could be infected with pure cultures of *Actinomyces bovis* when mixed with hog gastric mucin. Further work was undertaken to develop a quantitative method to determine the effect of aureomycin and penicillin on such experimental infections.

MATERIALS AND METHODS

Organisms.—A strain of *Actinomyces bovis*, designated "Goldin," was used. The basis for the choice was: uniform pathogenicity, size and number of lesions, and consistently typical morphology. During the course of this work, another strain of *Actinomyces bovis*, isolated from a dental plaque, proved to be uniformly pathogenic for mice, and was used in a comparative series of tests. It is referred to as the "dental" strain.

From the Department of Bacteriology, University of Illinois, College of Medicine.
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To maintain a standard culture throughout these investigations, the following procedure was used. Both organisms were grown on Brewer's thioglycollate medium No. 135 (BBL). From this, they were cultured on brain-heart infusion agar (Difco), and subsequently inoculated into mice according to the technique of Meyer and Verges. Ten days later material from the mouse lesions was inoculated directly into Brewer's thioglycollate medium, and pure cultures were obtained without necessity of subculturing. A number of tubes of Brewer's media were inoculated from this culture, incubated, and stored at −30° C. One of these was withdrawn and used for each experiment, which insured, as far as possible, having a uniform culture throughout this work.

Inoculum, Minimum Infective Dose, and Route of Inoculation.—To prepare the standardized inoculum, a transfer was made from a tube of the frozen stock culture to Brewer's medium and incubated four to five days. The culture was then transferred to the surface of brain-heart infusion agar contained in eight-ounce bottles and incubated in a Brewer's jar. The growth varied from rough, irregular, discrete colonies adherent to the medium to smooth, softer, and easily emulsified colonies. This was washed off the surface with a minimum amount of saline, and, when necessary, a wire was used to scrape off the adherent colonies. From this point on, to prevent reduction in viability of the organisms by aerobic conditions, the entire procedure was carried out as rapidly as possible. The saline suspension was centrifuged for a few minutes, and the sediment removed to a sterile mortar and ground gently. It was then diluted with saline to about double the former volume and filtered through a loose pledget of cotton. The filtrate was centrifuged at 2,000 r.p.m. (1,021 times gravity) for fifteen minutes in Hopkins tubes, and the packed cells diluted with saline to make a 2 per cent suspension. One part of the 2 per cent suspension plus one part of 5 per cent mucin* was then mixed, and 0.5 ml. injected intraperitoneally into mice. The 2 per cent suspension plus mucin was chosen after experimental data showed that 0.5 ml. of this concentration injected intraperitoneally was the minimum amount producing infection in 100 per cent of the mice. This was the routine inoculum used throughout the experimental procedures.

One series of four mice was injected intravenously with 0.2 ml. of this inoculum. Two of them died within three days with massive invasion of the thorax, abdomen, and cervicofacial region; in addition, all developed suppurating lesions at the site of inoculation in the tail. The portion of the tail distal to the lesions was gangrenous. This route of inoculation was discontinued, because the effect of intravenous mucin was unknown, and the widespread infection throughout the body made evaluation of therapy difficult.

Mice.—Young, male albino mice (Harlan Farms) weighing 10 to 15 Gm. were used throughout this work. Heavier and older mice gave erratic infectivity and were not used.

Control of Infection.—In order to confirm the infection in the animals, the following procedures were carried out on material from the lesions: gram stains and mounts in KOH-glycerin solution were made; material was streaked on brain-heart infusion agar, on blood agar, and Brewer's media was inoculated. During the first half of this work these procedures were carried out routinely on all infected mice. In all cases, except as noted in treated mice, the organism *Actinomyces bovis* was reisolated in pure culture and the gram stains and KOH mounts showed typical morphology. Acid-fast stains were consistently negative. Secondary bacterial invasion of the necrotic tissue at the site of the actinomycotic lesions could not be considered a factor in the progress of the disease.

In Vitro Sensitivities and Serum Concentrations.—In vitro studies on the sensitivity of the organisms to aureomycin were unsatisfactory due to two principal reasons: (1) the prolonged incubation time of the *Actinomyces bovis* strains used, and (2) the rapid deterioration of aureomycin in vitro.[9, 10] There are several aureomycin assay methods reported with four, eighteen, and twenty-four hour incubation periods claiming accuracies varying from 7.5 per cent to plus or minus 100 per cent.[11-15] It was thought that some of these methods may

*Hog gastric mucin, type 1701-W from Wilson Laboratories, Chicago, lot No. 73482 was used. A 5 per cent suspension of the dried mucin was made in saline with aid of Waring blendor, autoclaved at 15 pounds pressure for fifteen minutes, and then adjusted to pH 7.3 with sterile 1N NaOH. The mixture was cultured for sterility and kept at 4°C.

be used for *in vitro* sensitivity tests provided that the incubation time of *A. bovis* (Goldin) could be shortened. This strain was subcultured serially in thioglycollate broth at the first indication of growth for a period of four weeks. Eventually this strain changed from a granular growth requiring four to five days to a more diffuse type apparent in twenty-four hours. This growth was judged sufficient to attempt an in vitro aureomycin sensitivity test. The serial-tube dilution method and the tube-time exposure method were both attempted, but gave inconclusive and variable results.

Penicillin sensitivity tests on the original *Actinomyces bovis* (Goldin) strain by the tube dilution method disclosed a sensitivity of 0.05 unit per milliliter.

Determination of aureomycin and penicillin serum concentrations in the mice was not attempted because a survey of the literature indicated data of this nature would be meaningless.[16-20]

EXPERIMENTAL RESULTS

Evolution of the Disease.—Before in vivo evaluation of therapy could be done, information on the mode of evolution of the disease in mice had to be obtained. The dissemination of the disease was demonstrated by infecting a large series of mice and sacrificing them at intervals to check the progress of the lesions. The general course of the disease is as follows: the viscera usually showed lesions by the fifth day, and active invasion, as evidenced by daughter lesions, continued until about the twentieth to twenty-fifth day when a status quo appeared to be reached. From then on, until the last series of mice were autopsied at the end of the fourth month, the disease was chronic—the lesions became more circumscribed, the material within them heavier and more granular, and a few retrogressed, slowly becoming sterile and fibrous. Only a few mice died after the twenty-fifth day; and these deaths could not be attributed with certainty to actinomycosis, although it must have been a contributing factor. Only a small number of mice, an over-all average of 10 per cent, became inactive and died from two to ten days after infection with a massive invasion of nearly all organs.

On rare occasions a mouse would develop a cervicofacial actinomycosis with the only physical sign being a large tumorous mass under the skin along the mandible. Roughness, dullness, or loss of pelage was not noticeable. Throughout the experimental procedures no physical signs or symptoms could be correlated with the degree of infection of the mice.

A description of the lesions in detail is as follows: the visceral lesions were tubercle-like in appearance and usually 1 to 10 mm. in diameter, white to cream in color, firm, and filled with a thick mucoid purulent material. Sulfur granules were generally present, often large enough to be seen macroscopically. The sites of the lesions in descending order of occurrence were as follows: liver, liver-diaphragm area, inguinal lymph nodes, omentum, spleen, peritoneum, kidney, thoracic, and cervicofacial regions. The lesions were generally raised and rounded above the surface, quite symmetrical, and extended well into the underlying tissue. The tubercles could not be shelled out of the surrounding tissue Lesions were often visible on the cut surface of liver and spleen.

Progressive disease was evidenced by confluence of lesions, daughter tubercles and soft spreading necrosis. Healing was shown by circumscribed drying tubercles, fibrosis, and adhesions. However, it must be emphasized that progression or regression of the lesions was not clear cut in any significant propor-

tion of the cases. In the few mice that died within five days post inoculation, the abdominal organs, especially the liver and spleen, were covered with a white film of growth, and the surface of the organ was inflamed with patches of beginning necrosis. Also, at this early date, there were some small, soft, spreading lesions that were not imbedded in the tissue.

Later in the progression of the disease, quite often a portion of the liver or spleen became a solid white mass of friable, nonviable infected tissue. Occasionally an organ would be covered with multiple pin-point lesions. Histopathologic sections were taken from infected liver, lung, spleen, diaphragm, kidney, and cervicofacial lesions. These sections did not give a definitive picture, which may have been due to the small number of sections (15) which were examined. In general, the microscopic picture evidenced suppuration, chronic inflammatory cells, fibroblasts, and giant cells, along with a diffuse invasion of the tissue by the filamentous *Actinomyces bovis*. Sulfur granules in the tissue were sometimes visible.

Criteria of Infection and Therapeutic Effectiveness.—Because knowledge of the histologic changes in the mouse tissues due to actinomycosis was limited, the variation of the disease process during treatment could not be estimated as well as the quantity of disease change. No graded evaluation of the relative pathologic importance of the lesions in one organ as compared to another could be made. Relative mortality rates as a criterion could not be used, since the time of survival was altered by the fact that this disease is a chronic one in most cases. Staining and culture for the presence, viability, and invasiveness of the organism in the various types of lesions encountered would at best be a clumsy method of therapeutic evaluation because of the limitations imposed by the cultural and morphologic character of the actinomyces.

It was learned early in the work that treatment of infected animals by various therapeutic regimens resulted in only a small percentage of the mice being completely cleared of lesions. Therefore, a criterion of the degree of infection was a problem, since neither the histopathologic picture, gross appearance of the lesions, physical symptoms of the mouse, death, nor cure were available for the comparison of treated and untreated mice.

Various schemes for the numerical evaluation of the disease process were tried, and patterned after the work of Raleigh and Youmans[21] and Feldman[22] on tuberculosis. Due to the difficulties pointed out previously, a simple method involving only the amount of the morbid process present—the frequency and size of the lesions—could be used. The millimeters of lesions in the treated mice compared with the millimeters of lesions in the controls constituted the evidence for therapeutic efficiency.

The final method developed was as follows: the number of lesions in an organ times their diameter in millimeters gave a numerical value for each. Such calculations were made for all infected organs and added to give the total millimeters of lesions in each mouse. If an organ was studded with miliary lesions, an arbitrary value of 20 mm. was taken as the estimate for that organ. The lesions were usually circular, but in case of irregularities the longest axis was measured. Only lesions visible without dissection of the organs were counted.

since any other approach was impractical. The total millimeters of lesions for all the mice in the control series were compared with the total millimeters of lesions in the treated series. See Table I for an example of the evaluation of one mouse.

TABLE I: EXAMPLE OF EVALUATION OF INFECTION

Autopsy #174, cage 36, Goldin
No therapy. 10 days post infection.

LOCATION OF LESIONS	LESIONS		MM. OF LESIONS
	NUMBER	SIZE	
Liver	Miliary, entire		20*
Inguinal lymph node	1	2 mm.	2
	2	5 mm.	10
Omentum	2	2 × 3 mm.	6
	3	1 mm.	3
Spleen	1	4 mm.	4
	2	2 mm.	4
	1	1 mm.	1
Kidney	2	2 mm.	4
		Total lesions	54 mm.

*Since the pathologic importance in an organ of many miliary lesions as compared to several larger lesions could not be ascertained, the arbitrary value of 20 mm. was chosen in these incalculable miliary cases as representing approximately the half-way point between the lowest and highest value found in any one organ throughout the entire experiment.

Prophylactic Treatment of Actinomycosis in Mice.—Since penicillin is the first choice for treatment of actinomycosis today,[23] it was used as a control for comparative purposes. Table II, series 2 shows the results of penicillin treatment using Park, Davis penicillin S-R. This is a combination of procaine penicillin-G with buffered crystalline sodium penicillin-G which in aqueous solution is intended for intramuscular injection. That the prolonged effect of this preparation in human beings may not be true in mice is recognized and accepted as one of the possible sources of error in this work. A concentrated solution of the penicillin was made and kept refrigerated for no longer than one week. An aliquot portion was removed and diluted with normal saline for each day's supply.

In preliminary experiments, penicillin dosage was based on human therapy of 800,000 units per day. Results were poor and the dosage was increased to, and based on, 12,200,000 units per twenty-four hours, as recommended by Sanford and Barnes in their treatment of human cases.[24] The mice then received (Table II, series 2) 4,000 units every twenty-four hours, given intramuscularly in a 0.1 ml. dose. Treatment was started immediately after the animals were inoculated with *Actinomyces bovis.*

In series 3 of Table II are results of treatment with aureomycin. Aureomycin, Lederle, for oral administration was used. The mice received 2 mg. per day which would be equivalent to 6 Gm. per day in man. The powder was dissolved in distilled water, diluted to the proper concentration for use daily, and stored at 0° C. between treatments. The mice received 1 mg. per dose in a total volume of 0.5 millimeter. This was administered via esophageal intubation with a 4 cm. section of a No. 6 (French) ureteral catheter placed over the needle of a hypodermic syringe. The two daily doses were given at eight-hour intervals.[25] Treatment started immediately after the animals were inoculated

TABLE II. COMPARATIVE PROPHYLACTIC TREATMENT SERIES

MOUSE	MM. OF LESIONS: LIVER	LIVER DIAPHRAGM	INGUINAL L.N.	OMENTUM	PERITONEUM	SPLEEN	KIDNEY	C-F TH.	TOTAL MM.	DAY OF DEATH	CAUSE DEATH*
Series 1: Goldin, control, no therapy											
1	18	20	15	10	5	2	0	0	70	10	S
2	22	25	20	14	5	20	4	10	120	9	A
3	20	31	18	4	10	15	0	0	98	10	S
4	26	15	19	12	0	0	0	0	72	10	S
5	25	30	5	9	6	10	4	0	89	10	S
Total mm.	111	121	77	49	26	47	8	10	*449*		
Series 2: Goldin, penicillin S-R, 1 dose, 4,000 units per day, prophylactic											
1	22	14	10	2	2	4	2	0	56	10	S
2	24	10	19	12	0	0	0	0	65	10	S
3	10	15	5	2	4	3	1	0	40	10	S
4	10	16	4	3	6	3	0	0	42	10	S
5	18	5	15	10	2	2	1	1	54	10	S
Total mm.	84	60	53	29	14	12	4	1	*257*		
									P = < .02 when compared with series 1†		
Series 3: Goldin, aureomycin (capsules), orally 2 mg. per day, prophylactic											
1	10	14	8	5	3	2	2	0	44	10	S
2	4	0	10	0	2	3	0	0	19	10	S
3	8	12	4	5	3	2	5	0	39	10	S
4	2	7	4	2	4	0	0	0	19	9	E
5	8	15	3	0	0	0	0	0	26	10	S
Total mm.	32	48	29	12	12	7	7	0	*147*		
									P = < .01 when compared with series 1†		
									P = < .02 comparing series 2 & series 3 (Snedecor, p. 76) P values less than .05 indicate a statistically significant difference.		

*S—sacrificed; E—experimental; A—actinomycosis (?).
†Snedecor, p. 83.

with the organism. It was interesting to note that the mice in which the acid aureomycin was introduced directly into the esophagus showed no evidence of toxicity, physical damage, or malnutrition other than a slower gain in weight and a more liquid feces.

The oral capsules of aureomycin used here proved to be unsatisfactory due to insoluble vehicle substances used. It is questionable whether the dosage was as accurate as desired.

In Table II it will be noted that the total millimeters of lesions in the control series is 449 as compared to 147 in the aureomycin and 257 in the penicillin treated series. A statistical analysis was made of these data following procedures as outlined by Snedecor.[26] In all cases the difference between those treated and the controls, in our estimation, was found to be significant at the 0.05 level. In the Table II analysis it will be noted that the aureomycin has a slight statistical advantage over penicillin.

In an effort to improve the results of therapy over that described above, the treatment was altered as follows: the type of penicillin was changed to Wycillin Fortified (Wyeth). This is essentially the same product as used previously, but it appeared that the particle size was smaller, giving a more uniform suspension in the concentrations used here. The penicillin dosage was exactly the same unitage as administered previously, but was divided into two treatments at twelve-hour intervals using alternate leg muscles as the site of injection.

The use of oral capsules of aureomycin was discontinued, and crystalline aureomycin hydrochloride standard with a potency of 996 gamma per mg. (Lot No. 7-9726) provided by Lederle Laboratories was substituted. The oral dosage was greatly increased and was comparable to 500 mg. per kg. per day in man as given by Harrell and associates.[19] Each mouse received 7.5 mg. per day. This amount was also administered via esophageal intubation in 0.5 ml. doses every eight hours for a period of fifteen days.

TABLE III. PROPHYLACTIC SERIES, DENTAL STRAIN, AUREOMYCIN 7.5 MILLIGRAMS PER DAY, ORALLY, SACRIFICED FIFTEENTH DAY

	NO. OF MICE	TOTAL MM. OF LESIONS	MEAN AND RANGE	PER CENT OF MICE FREE OF LESIONS	PROBABILITY VALUE WHEN COMPARED WITH CONTROL
Series 1 Control	10	406	40.6 19 to 64	0	
Series 2 Aureomycin	10	34	3.4 0 to 9	30	<.01

This altered therapeutic regimen with aureomycin was used in a series of mice infected with *Actinomyces bovis* (Dental). Treatment was again instituted immediately after inoculation with the organisms. This series gave more definite results as shown in Table III. It will be noted that some 30 per cent of the treated animals were completely cleared of lesions.

In the next group of mice, Table IV, series 1, 2, and 3, the penicillin therapy (Wycillin) was as last described. However, the aureomycin therapy was al-

tered to conform to the technique of Brainerd.[16] In this routine, two intravenous injections of aureomycin were given the first twenty-four hours only, in addition to the same oral dose as previously used. According to Brainerd, and Sanders,[27] the intravenous aureomycin brings the serum concentrations to effective levels rapidly to supplement the slower absorption rate of oral administration. The intravenous aureomycin used was the standard market product (Lederle Laboratories) with l-leucine 2.6 per cent as the solvent. Each mouse was given 0.5 mg. in two doses of 0.25 mg. each in 0.1 ml. of solution.

TABLE IV. COMPARATIVE PROPHYLATIC SERIES, GOLDIN STRAIN, SACRIFICED ON FIFTEENTH DAY

	NO. OF MICE	TOTAL MM. OF LESIONS	MEAN AND RANGE	PER CENT OF MICE FREE OF LESIONS	PROBABILITY VALUE WHEN COMPARED WITH CONTROLS
Series 1 Control	14	464	33 13 to 61	0	
Series 2 Penicillin 4,000 U./day	15	64	4.3 0 to 15	26.6	<.01
Series 3 Aureomycin 7.5 mg./day orally, plus I.V. dose initially	13	102.5	8 0 to 21	7.7	<.01

It will be noted in the Table IV analysis that 26.6 per cent of the penicillin treated mice and 7.7 per cent of the aureomycin treated mice were completely cured. The results obtained nevertheless indicated no statistical difference between the two modes of therapy.

Treatment of Established Infection.—In order to obtain some data on the efficacy of aureomycin treatment on fully developed actinomycosis, a series of mice were infected and allowed to develop the disease for ten days before treatment was instituted. The aureomycin therapy was the same at last described.

TABLE V. THERAPEUTIC SERIES, GOLDIN STRAIN, AUREOMYCIN ONLY

	NO. OF MICE	TOTAL MM. OF LESIONS	MEAN AND RANGE	PER CENT OF MICE FREE OF LESIONS	PROBABILITY VALUE WHEN COMPARED WITH CONTROLS
Series 1 Control, sacrificed 10th day	14	455	32.5 0 to 86 (4 mice below 25)	7 (1 mouse)	
Series 2 Control, sacrificed 25th day	13	750	50.5 19 to 85 (2 mice below 30)	0	
Series 3 Aureomycin from 10th to 25th day, when sacrificed	10*	161	16.1 0 to 60	30	<.01 (25 day control) <.05 (10 day control)

*Five mice of original fifteen died before therapy was instituted and were not included in data.

The data are summarized in Table V, series 1, 2, and 3. It will be seen that there was a 51 per cent increase in the millimeters of lesions between the tenth day controls and the twenty-fifth day controls. At the same time there was a 57 per cent decrease in the millimeters of lesions between the ten day controls and the mice that were treated from the tenth day to the twenty-fifth day. At the end of the treatment period, 30 per cent of the mice were cleared of lesions.

From these results it appeared that a ten to fifteen day period of treatment was adequate to evaluate the effectiveness of antiactinomycotic agents. However, in this instance, to complete the data, a series of five mice were treated with aureomycin for a full twenty-five days starting after a ten day incubation period. These mice were found to be free of lesions, but had residual scars and adhesions as evidence of previous infection. The controls for this series had a mean of 68 millimeters of lesions.

DISCUSSION

The standardization procedure for the inoculum developed in this work is reproducible and as accurate as possible with these organisms. A microscopic examination of the 2 per cent suspension revealed single organisms as well as small clumps, the latter containing from three to twenty organisms. The fact that both the dental and Goldin strains had the same minimum infective dose was a fortuitous circumstance. This concentration probably could not be applied to all strains of *Actinomyces bovis*.

The simple method of evaluating the extent of the disease process, utilizing number and size of lesions, leaves much to be desired. More accurate work must await greater knowledge of this infection in mice. The description of lesions and progression of the disease presented here can be considered only as groundwork for further experimental studies. The microscopic pathology, especially, must be more clearly delineated in order to detect small modifications due to therapy.

When the treatment of actinomycosis was instituted immediately after injection of the organisms, both aureomycin and penicillin exhibited a prophylactic effect by preventing infection completely in a small percentage of the mice and inhibiting the development of the disease in the remainder. It has been adequately demonstrated that aureomycin is widely distributed in body fluids and tissues, and hence should be in contact with the actinomyces in all locations.[28, 29]

No attempt has been made to show the superiority of one of these agents over the other in a conclusive manner from these limited animal experiments. One of the primary purposes of this work was to obtain information on the in vivo activity of the relatively new antibiotic aureomycin against these organisms. The results do indicate that aureomycin has the same order of effectiveness as penicillin in mice. This is borne out by three cases of clinical actinomycosis, reported near the completion of this work, in which two were resistant to penicillin but were cured with aureomycin.[30-32] Although a small percentage of mice was cured, the data indicate that both these drugs have largely a suppressive effect on the disease.

Why the disease became chronic, the ultimate results of the infection beyond four months' duration, and the reason why older and heavier mice gave erratic infectivity were not investigated. Future work may show whether or not these points, and the therapy of actinomycosis, are affected by strong immunological factors.

CONCLUSIONS

Standardization of the minimum infective dose for 100 per cent of the animals was accomplished using two strains of *Actinomyces bovis*.

The expected dissemination and progression of the disease process in mice was outlined.

A method was devised to evaluate the extent of infection and the efficiency of therapeutic agents in the treatment of this disease.

The prophylactic and therapeutic effectiveness of aureomycin and penicillin against actinomycosis of mice was demonstrated.

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THE ACUTE EFFECTS OF PARATHYROID HORMONE ON THE METABOLISM OF ENDOGENOUS PHOSPHATE

CHARLES R. KLEEMAN, M.D.,* NEWINGTON, CONN., AND ROBERT E. COOKE, M.D.,** NEW HAVEN, CONN.

THE site and mechanism of action of parathyroid hormone has been a controversial issue for over two decades. However, it appears well established that the injection of a potent parathyroid extract or hyperfunction of the parathyroid glands leads to the following metabolic changes: (1) rise in urinary phosphorus excretion, (2) fall in serum phosphorus, (3) rise in serum calcium, and (4) rise in urinary calcium excretion. Removal or hypofunction of the parathyroid glands leads to the converse of the above.[1-12] Accepting these changes, there remains to be decided the site of action of the hormone that leads to the above and which of these changes are the direct or acute effects, and which are the indirect or remote end results.

By far, the majority of studies point to a direct action of parathyroid hormone on the renal excretory mechanism for phosphorus.[1-13, 20, 21, 22, 24, 35] There are concrete objections to the primary calcium effect theory[11, 14-18, 23, 40] and with but a few exceptions[11, 16] the latter does not appear to withstand critical analysis. In almost all experiments[11, 17, 18] the urinary and serum changes in calcium are gradual and lag behind the abrupt changes in phosphorus excretion. It is with these abrupt changes in phosphorus excretion after parathyroid hormone, and the renal mechanism involved, that the present paper is concerned.

Great confusion appears to exist regarding both the normal mechanisms of phosphorus excretion and the action of parathyroid hormone on these mechanisms. Some of the conflicting results are due to differences in the amount of time elapsed between the administration of the hormone and the measurement of its renal effect.

The great importance of time in these studies has been stressed by others,[1, 3] and is demonstrated in the present paper. In addition, some experiments have been concerned with the renal mechanism for the excretion of endogenous phosphate, while others have been complicated by the addition of exogenous phosphate.[14, 15, 17]

From the Veterans Administration Hospital, Newington, Conn., the Department of Medicine, Yale University School of Medicine, and the Metabolic Study Unit, Department of Pediatrics, Yale University School of Medicine, New Haven, Conn.

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*Senior Resident in Medicine, Veterans Administration Hospital.

**Instructor in Pediatrics, Yale University School of Medicine.

The data of Ollayas, Winkler and associates[33, 34] and Govaerts[36] indicate that the renal mechanism for the excretion of endogenous phosphate may be entirely different from that noted after large phosphate loading experiments.

The present studies on the mechanism of action of parathyroid hormone on endogenous phosphorus were stimulated by the already existing controversies as stated above, and by the demonstration in an early single experiment[45] that the acute effects of intravenous parathyroid hormone, used for diagnostic purposes in a case of renal hypercalciuria, may be followed by rebound effects in which less phosphorus is excreted than before the administration of parathyroid hormone.

METHODS

Subjects used in the present study were normal adult men ranging in age from 25 to 30 years, three children, ages 3, 7 and 11 years, all with chronic renal disease, and two postthyroidectomy hypoparathyroid women patients, ages 65 and 45 years. All studies were done in a postabsorptive state beginning in the morning around 8:00 A.M. The subjects carried out routine daily activities during the experiments. Two hundred and three hundred milliliters of water was ingested at hourly intervals. Each patient served as his own control, the control periods being approximately one hour in length, three to four in number. During these periods, in most experiments, urines were collected under oil. At the end of the control period 200 units of parathyroid hormone were given intravenously to adults and 50 to 100 units intravenously to children. Approximately hourly urines were collected subsequent to this; in some cases one-half hour specimens were collected immediately after administration of the hormone. The entire experiment lasted approximately eight hours, and four to five arterialized blood specimens were taken under oil from the dorsum of the hand (which had been submerged in hot water for ten minutes). Serum calcium,[48] phosphorus,[49] bicarbonate,[50] sodium,[51] potassium,[52] chloride,[53] and creatinine concentrations were determined. Serum water was determined by weighing one milliliter of serum before and after drying at 100° C. In some cases urine pH was determined by the Beckman pH meter. The rate of glomerular filtration was measured by endogenous creatinine clearances, using the Hare and Hare[46] modification of the Borsook adsorption technique for true creatinine chromagen. In all urines, phosphorus[49] and calcium[48] were determined; in some, sodium,[51] potassium,[52] chloride[53] ammonia,[54] titratable acid,[55] and bicarbonate content[56] were also measured.

RESULTS

(*a*) *Phosphorus Excretion:* Following the intravenous administration of parathyroid extract to the normal subjects, there occurred a two to over fourfold increase in minute phosphorus excretion above the control period levels. This increase appeared to reach its peak during the first and second hours in most cases, but as late as the third hour in one case and as early as the first half-hour in another. (Table I, Experiments 1 through 6).

In Experiment R. A., Table II, with mild renal tubular impairment, the increase was not quite as great as the six experiments in individuals with normal renal function. In marked contrast to the above experiments are those performed in the two cases with severe renal impairment, Table II, Experiments F. M. and Boy B. In these cases there was little or no increase in the minute excretion of phosphorus. The two hypoparathyroid subjects showed

TABLE I. DEMONSTRATING EFFECTS OF INTRAVENOUS PARATHYROID EXTRACT ON THE RENAL MECHANISM OF PHOSPHORUS EXCRETION

PERIOD	DURATION OF PERIOD (MIN.)	SERUM PHOSP. MM/L. OF SERUM H_2O	SERUM CALCIUM (MG. %)	CREATININE CLEARANCE	URINE VOLUME (C.C./MIN.)	PHOS. EXCRETED (MICROM/MIN.)	PHOS. FILT. (MICRO M/MIN.)	PHOSP. REABS. (MICRO M/MIN.)	PHOSP. REABS. (% OF FILT. LOAD)	PHOSP. REABSORBATE CONC. (MM/L.)
EXPERIMENT 1—C. K. (1)										
Pre 1	60	1.168 (3.30)†	10.4	123.0‡	4.4	7.65	143.7	136.1	94.7	1.149
Pre 2	60	1.168		123.0	3.9	5.67	143.7	138.0	95.1	1.158
Pre 3	60			123.0	5.8	7.17	143.7	136.5	95.0	1.163
(Parathyroid Extract 200 units I. V.)										
Post 1	30	(3.40)†		124.0	5.1	14.34	144.9	130.6	90.2	1.100
Post 2	30			124.0	3.3	23.2	144.9	121.7	84.0	1.009
Post 3	30	1.168 (3.30)†		124.0	3.6	21.7	144.9	123.2	85.1	1.022
Post 4	30			124.0	5.5	22.0	144.9	122.9	84.8	1.035
Post 5	30		12.0	124.0	4.2	14.0	144.9	130.9	90.4	1.091
Post 6	60	1.209 (3.60)†		124.0	4.4	14.0	149.9	135.9	90.6	1.135
Post 7	60		11.9	124.0	2.0	12.7	149.9	137.2	91.5	1.125
Post 8	240			124.0	0.7	11.5	149.9	138.4	92.3	1.121
EXPERIMENT 2—B. C. (1)										
Pre 1	60			146.0‡	9.5	16.0	189.4	173.4	91.5	1.271
Pre 2	60	1.392 (3.8)†	9.6	146.0	4.8	17.0	189.4	172.4	91.0	1.220
Pre 3	60			146.0	7.4	22.5	189.4	166.9	88.1	1.202
(Parathyroid Extract 200 units I. V.)										
Post 1	60			140.0	5.2	24.5	194.9	170.4	87.4	1.265
Post 2	30	1.392 (4.2)†		140.0	3.3	32.0	194.9	162.9	83.6	1.191
Post 3	30			140.0	5.2	31.3	194.9	163.6	83.9	1.212
Post 4	30			140.0	5.8	23.7	194.9	171.2	87.9	1.272
Post 5	30	(3.8)†	11.9	140.0	0.8	18.01	194.9	176.9	90.9	1.269
Post 6	60	1.347		140.0	1.9	11.05	188.5	177.5	94.3	1.285
Post 7	60			140.0	4.4	11.0	188.5	177.5	94.3	1.310
Post 8	240	(3.9)†	12.5	140.0	0.5	5.54	188.5	183.0	97.2	1.314
EXPERIMENT 3—MR. A.										
Pre 1	60	1.075 (3.12)†		125.5	0.95	8.64	144.0	135.4	94.0	1.086
Pre 2	60	1.147*	9.3	128.5	1.5	12.68	142.4	129.7	91.1	1.021
Pre 3	67	1.220 (3.52)†	9.7	111.5	9.3	18.34	128.0	109.7	85.6	1.074
(Parathyroid Extract 200 units I. V.)										
Post 1	53			115.0	0.77	28.32	136.1	107.8	79.1	0.944
Post 2	60			114.0	0.75	28.45	134.8	106.4	79.1	0.939
Post 3	60	1.146 (3.32)†	9.1	116.7	7.7	43.90	138.0	94.1	68.2	0.863
Post 4	55	1.145*		103.8	9.9	21.52	119.0	97.5	81.9	1.038
Post 5	125	1.244	10.3	107.8	8.1	26.52	123.4	96.9	78.5	0.972
Post 6	120	(3.60)		112.8	5.5	29.54	129.1	99.6	77.1	0.929
EXPERIMENT 4—MR. B.										
Pre 1	60	1.172 (3.64)†		137.7	0.37	4.78	162.7	157.9	97.1	1.147
Pre 2	61	1.181	10.0	145.8	0.36	5.16	172.2	167.0	97.1	1.149
Pre 3	66	1.190 (3.44)†	10.9	133.0	0.35	6.79	157.1	150.3	95.7	1.133

TABLE I—CONT'D

PERIOD	DURATION OF PERIOD (MIN.)	SERUM PHOSP. MM/L. OF SERUM H_2O	SERUM CALCIUM (MG. %)	CREATININE CLEARANCE	URINE VOLUME (C.C./MIN.)	PHOS. EXCRETED (MICROM/MIN.)	PHOS. FILT. (MICRO M/MIN.)	PHOSP. REABS. (MICRO M/MIN.)	PHOSP. REABS. (% OF FILT. LOAD)	PHOSP. REABSORBATE CONC. (MM/L.)
(Parathyroid Extract 200 units I. V.)										
Post 1	53			105.0	0.26	10.53	124.0	113.5	91.5	1.084
Post 2	88			134.0	1.29	14.74	158.2	143 5	90.8	1.081
Post 3	47	1.110 (3.20)†	10.1	100.5	4.00	10.31	118.7	108.4	91.4	1.123
Post 4	45			157.6	7.33	13.70	186.1	172.4	92.7	1.146
Post 5	120			131.3	1.81	9.12	155.1	146.0	94.1	1.127
Post 6	120	1.315 (3.80)†	10.6	125.6	1.63	12.38	148.4	136.0	91.7	1.096
EXPERIMENT 5—C. K. (2)										
Pre 1	60	1.34 (3.84)†	10.2	130.2	6.76 c.c.	9.83	174.3	164.5	94.4	1.33
Pre 2	60	1.35*		121.0	4.95 c.c.	6.13	163.3	157.2	96.2	1.35
Pre 3	91	1.37 (3.92)†	10.3	117.4	5.55 c.c.	9.68	158.4	148.7	93.8	1.33
(Parathyroid Extract 200 units I. V.)										
Post 1	30	1.31*		144.3	6.40 c.c.	34.5	189.1	154.6	81.7	1.12
Post 2	60			118.2	6.63 c.c.	18.7	154.8	136.1	87.9	1.22
Post 3	60	1.26 (3.60)†		119.3	6.61 c.c.	21.4	154.0	132.6	86.1	1.17
Post 4	60	1.29*		122.9	2.38 c.c.	14.8	158.6	143 8	90.8	1.19
Post 5	60	1.33 (3.80)†	10.4	123.0	4.05 c.c.	15.5	158.6	143.1	90.1	1.20
Post 6	165			116.1	2.47 c.c.	12.6	149.7	137.1	91.5	1.21
EXPERIMENT 6—B. C. (2)										
Pre 1	60	1.48*	10.6	143.5	10.31	10.63	212.3	201.7	95.0	1.51
Pre 2	59	1.43 (4.10)†		144.8	4.61	10.85	214.2	203.4	95.0	1.45
Pre 3	64	1.54 (4.40)†	10.9	150.6	8.28	16.72	222.9	206.2	92.6	1.45
(Parathyroid Extract 200 units I. V.)										
Post 1	57	1.53*		138.5	1.89	39.3	211.9	172.6	81.5	1.26
Post 2	62			152.6	7.52	30.34	233.4	203.1	87.0	1.40
Post 3	55	1.53 (4.40)†		143.1	4.80	22.30	219.1	196.8	89.8	1.42
Post 4	63	1.51*	10.5	125.0	1.92	16.84	188.7	171.9	91.1	1.40
Post 5	60	1.50 (4.30)†		137.0	1.32	13.40	207.9	194.5	93.6	1.43
Post 6	185			146.5	1.05	13.45	219.8	206.4	93.9	1.42

*Average values of serum phosphorus determinations.
†Serum phosphorus mg. per cent.
‡Average values for creatinine clearance from Experiments 5 and 6.

responses qualitatively similar to the normals, but quantitatively greater responses amounting to over sixfold increases in phosphorus excretion. Table III, Experiments 7 and 8.

(b) Glomerular Filtration Rate, Serum Electrolyte Levels, and Filtered Loads: Throughout the pre- and postparathyroid periods the glomerular filtration rate showed no consistent or significant changes. The slight variation in filtration rate noted could well be ascribed to the fact that the experiments were performed while the subjects were ambulatory with varying degrees of mild physical activity. Unfortunately, in Experiments 1 and 2, Table I, due to

TABLE II. DEMONSTRATING EFFECTS OF PARATHYROID EXTRACT ON MECHANISM OF PHOSPHORUS EXCRETION IN CHRONIC RENAL DISEASE

PERIOD	DURATION IN MINUTES	PHOSPHORUS MM/HR.	CALCIUM MM/HR.	SODIUM MM/HR.	POTASSIUM MM/HR.	CHLORIDE MM/HR.
EXPERIMENT R. A. (AGE 7 YEARS)						
Pre 1	60	0.836	0.147	2.4	2.7	3.1
Pre 2	60	0.838	0.162	1.7	3.6	3.1
Pre 3	60	0.916	0.181	1.8	3.7	3.1
Pre 4	60	0.965	0.160	1.6	3.3	2.4
(Parathyroid Extract 50 units I. V.)						
Post 1	60	1.398	0.227	3.3	2.5	3.9
Post 2	60	1.616	0.176	2.4	2.5	2.9
Post 3	60	1.577	0.197	2.5	2.2	3.0
Post 4	60	1.496	0.257	2.2	4.4	3.0
Post 5	60	1.432	0.253	2.9	3.3	2.8

PERIOD	DUR. OF PERIOD (MIN.)	SERUM PHOSP. (MM/L. OF SERUM H_2O	SERUM CALCIUM MG. %	CREATININE CLEARANCE	URINE VOLUME (C.C./MIN.)	PHOSP. EXCRETED (MICRO M/MIN.)	PHOSP. FILT. (MICRO M/MIN.)	PHOSP. REABS. (MICRO M/MIN.)	PHOSP. REABS. % OF FILT. LOAD	PHOSP. REABSORBATE. CONC. (MM/L.)
EXPERIMENT F. M. (AGE 11 YEARS)										
Pre 1	60	2.52 (7.80)*	5.6	1.82	0.52	4.03	5.24	1.21	19.1	0.932
Pre 2	60			1.86	0.52	3.93	5.36	1.43	26.7	1.068
Pre 3	60			1.56	0.57	3.76	4.49	0.73	16.2	0.739
Pre 4	60	2.35 (7.28)*		1.57	0.70	3.81	4.20	0.39	9.3	0.449
(Parathyroid Extract 50 units I.V.)										
Post 1	60			1.89	0.70	3.81	5.05	1.24	24.5	0.960
Post 2	60			1.52	0.63	3.70	4.06	0.36	24.4	0.410
Post 3	60			2.60	1.22	6.80	8.27	1.47	17.8	0.930
Post 4	60	2.79 (8.64)*	5.9	1.73	0.67	4.08	5.51	1.43	26.0	1.349
Post 5	60			1.99	0.78	5.03	6.33	1.30	20.5	1.072
EXPERIMENT BOY B										
Pre 1	120			7.86	0.54	7.91	17.15	9.24	53.8	1.262
Pre 2	120	2.18 (6.3)*	6.3	4.49	0.37	4.58	9.80	5.22	52.2	1.268
(50 units of Parathyroid Extract I. V.)										
Post 1	120	2.21 (6.4)*	6.3	4.33	0.46	5.50	9.58	4.08	42.7	1.055
Post 2	120			3.94	0.52	5.33	8.71	3.38	38.8	0.990
Post 3	120	2.03 (5.9)*	5.4	6.88	0.53	5.00	13.95	8.95	64.1	1.410
Post 4	180				0.61	5.19				

*Serum phosphorus mg. per cent.

technical difficulties, we were unable to accurately calculate the glomerular filtration rates. Those given in these two experiments are the average of the pre- and postparathyroid periods as determined in these two subjects in another set of experiments, Table I, Experiments 5 and 6, under similar conditions.

As noted in Experiments 1 through 6, Table I, there were no significant changes in serum phosphorus levels, either before or after the administration of the parathyroid hormone. In general, similar results were noted in serum calcium determinations. It is very difficult to interpret the high values for

TABLE III. DEMONSTRATING THE EFFECTS OF INTRAVENOUS PARATHYROID EXTRACT ON THE RENAL MECHANISM OF PHOSPHORUS EXCRETION IN HYPOPARATHYROIDISM

PERIOD	DURATION OF PERIOD (MIN.)	SERUM PHOSP. (MM/L.) OF SERUM H_2O	SERUM CALCIUM (MG. %)	CREATININE CLEARANCE	URINE VOLUME (C.C./MIN.)	PHOSP. EXCRETED (MICRO M/MIN.)	PHOSP. FILT. (MICRO M/MIN.)	PHOSP. REABS. (MICRO M/MIN.)	PHOSP. REABS. (% OF FILT. LOAD)	PHOSP. REABSORBATE CONC. (MM/L.)
EXPERIMENT 7—MISS R (1)										
Pre 1	60	1.510	9.6	54.1	3.18	2.413	81.4	78.99	97.0	1.550
Pre 2	65			45.8	2.02	1.983	68.9	66.92	97.2	1.530
Pre 3	80	1.505		61.3	3.71	2.396	92.2	89.80	97.4	1.560
(Parathyroid Extract 200 units I. V.)										
Post 1	30	1.496		58.5	3.13	10.20	88.0	77.80	88.4	1.407
Post 2	30			46.2	1.03	11.56	68.9	57.34	83.2	1.270
Post 3	31	1.490		59.0	1.26	14.20	87.9	73.70	83.9	1.277
Post 4	32			62.7	4.13	14.83	93.4	78.57	84.1	1.342
Post 5	62	1.480		56.8	2.32	11.25	84.7	73.45	86.8	1.349
Post 6	60			53.2	2.13	10.19	78.3	68.11	87.0	1.332
EXPERIMENT 8—MISS C (1)										
Pre 1	58	1.71	8.7	80.6	1.60	6.72	137.8	131.1	95.1	1.660
Pre 2	61			76.2	4.66	6.75	130.4	123.6	94.8	1.727
Pre 3	59	1.71		68.4	4.86	7.29	117.0	109.7	93.7	1.728
(Parathyroid Extract 200 units I. V.)										
Post 1	31	1.71		83.2	6.42	20.8	133.1	112.3	84.3	1.462
Post 2	28			84.2	4.82	37.5	134.6	97.1	72.1	1.223
Post 3	31	1.60		87.2	8.96	39.7	139.5	99.8	71.6	1.293
Post 4	35			72.0	8.34	25.5	115.1	89.6	77.8	1.391
Post 5	61	1.50		73.8	8.13	20.4	118.0	97.6	82.6	1.485
Post 6	57		8.5	73.9	7.16	15.0	118.2	103.2	87.4	1.500

serum calcium seen in Experiments 1 and 2, for they were not seen in any of the other experiments or in those repeated in the same subjects. It is felt that as these two determinations were done at the same time, they most likely represent a technical error. Although not listed in the charts or tables, the serum sodium and potassium in the two experiments (1 and 2), in which their respective excretions were measured, showed no significant change. Serum chloride fell from 100 meq. per liter to 93 meq. per liter in subject C. K., and from 101 to 96 meq. per liter in subject B. C. These changes appeared to be due to slight changes in serum water.

As a result of the lack of significant change in either serum phosphorus level or glomerular filtration rate, the calculated filtered load of phosphorus showed no consistent change before or after parathyroid administration in any of the experiments.

(c) Electrolyte Excretion Other Than Phosphorus, Including Ammonia, Titratable Acid, and Urine pH Change: As electrolyte, ammonia, and titratable acid determinations were done in only two experiments, Table IV, Experiments 1 and 2, the significance of this data is difficult to interpret. In subject C. K., Experiment 1, there was a moderate fall in hourly excretion of sodium following parathyroid hormone. In subject B. C., Experiment 2, after an initial moderate rise in sodium excretion in the first post-parthyroid hour, it fell to lower levels as noted in subject C. K., Table I, Experiment 1. In both subjects similar changes were noted for potassium excretion, but to a slighter degree. Chloride

excretions also showed this trend in both subjects. Except for slight rises in calcium excretion in the first post-parathyroid period, hourly excretions in both subjects fell moderately below control periods. Although ammonia and titratable acids tended to fall slightly in the postparathyroid periods, the results both before and after hormone administration were so variable that no clear-cut effect of parathyroid extract could be deduced. Again, in regard to pH changes, Table V, the results were quite variable and, although they may be interpreted as showing slight trend toward the alkaline side in the immediate post-parathyroid period, there was no correlation between this effect and the periods of maximum phosphorus excretion.

TABLE IV. DEMONSTRATING EFFECT OF PARATHYROID EXTRACT ON THE RENAL EXCRETION OF PHOSPHORUS, OTHER ELECTROLYTES AND URINARY pH

		MM/HOUR EXCRETED							
PERIOD	DURATION	PHOSPHORUS	CALCIUM	SODIUM	POTASSIUM	CHLORIDE	TITRATABLE ACID	AMMONIA	pH
EXPERIMENT 1—C. K. (1)									
Pre 1	60	0.46	0.067	2.83	5.74	7.48	0.57	1.80	5.88
Pre 2	60	0.34		2.52	5.13	6.93	0.47	1.78	5.94
Pre 3	60	0.43	0.047	2.52	6.38	6.78	0.52	1.26	6.38
(Parathyroid Extract 200 units I. V.)									
Post 1	60	0.86	0.100	1.86	9.52	10.85	0.05	0.89	7.0
Post 2	30	0.69				2.72	0.05	0.28	6.95
Post 3	30	0.65		0.58	3.31	1.90	0.27	0.61	6.62
Post 4	30	0.66	0.010			1.92	0.45	0.80	6.45
Post 5	30	0.42		0.50	2.16	1.50	0.36	0.49	6.45
Post 6	60	0.84	0.010	1.39	3.75	3.31	0.79	1.34	6.21
Post 7	60	0.76	0.036	1.75	2.52	3.30	0.71	1.35	5.84
Post 8	240	2.76	0.20	6.61	7.51	10.62			
EXPERIMENT 2—B. C. (1)									
Pre 1	60	0.96	0.15	11.25	9.0	15.92	0.57	1.78	6.18
Pre 2	60	1.02		11.71	7.83	15.95	0.78	0.19	5.92
Pre 3	60	1.35	0.09	11.94	8.64	17.40	1.15	2.10	5.99
(Parathyroid Extract 200 units I. V.)									
Post 1	60	1.47	0.29	18.77	9.78	23.44	0.16	1.77	6.61
Post 2	30	0.96				7.34	0.08	0.33	6.95
Post 3	30	0.94	0.01	1.33	4.05	3.57	0.62	0.89	6.20
Post 4	30	0.83	0.02	0.84	3.94	2.94	0.59	1.07	6.05
Post 5	30	0.45				1.84	0.45	0.72	5.45
Post 6	60	0.69	0.04	1.99	3.18	4.31	0.82	1.76	5.60
Post 7	60	0.66	0.10	3.57	4.84	4.72	0.85	0.71	5.88
Post 8	60	1.33	0.04	6.89	4.02	11.03			

(d) "Net" Tubular Reabsorption of Phosphorus: In all experiments performed in the subjects with normal renal function, definite marked decreases in the "net" tubular reabsorption of phosphorus took place following parathyroid hormone administration, Table I and III. In the control periods approximately 88 to 98 per cent of the filtered loads of phosphorus were reabsorbed, and following the hormone this "net" decrease in tubular reabsorption of phosphorus resulted in approximately 7 to 22 per cent decreases in the amount of the filtered load reabsorbed. This is illustrated in Fig. 1.

In all cases this "net" decrease accounted for the great increases in minute excretion of phosphorus, Tables I and III, and the increases in phosphorus clear-

TABLE V. URINE pH CHANGES BEFORE AND AFTER PARATHYROID

C. K. (1)		B. C. (1)	MR. A.	MR. B.
Pre 1	5.88	6.18	5.70	5.13
Pre 2	5.94	5.92	6.62	5.19
Pre 3	6.38	5.99	6.50	5.46
		(200 units of Parathyroid Extract I. V.)		
Post 1	7.00*	6.61*	7.35	6.00
Post 2	6.95*	6.95*	5.77	5.40
Post 3	6.62*	6.20*	6.22	6.05
Post 4	6.45*	6.05*	6.30	6.52
Post 5	6.45	5.45	6.12	6.17
Post 6	6.21	5.60		
Post 7	5.84	5.88		

* ½ hour periods.

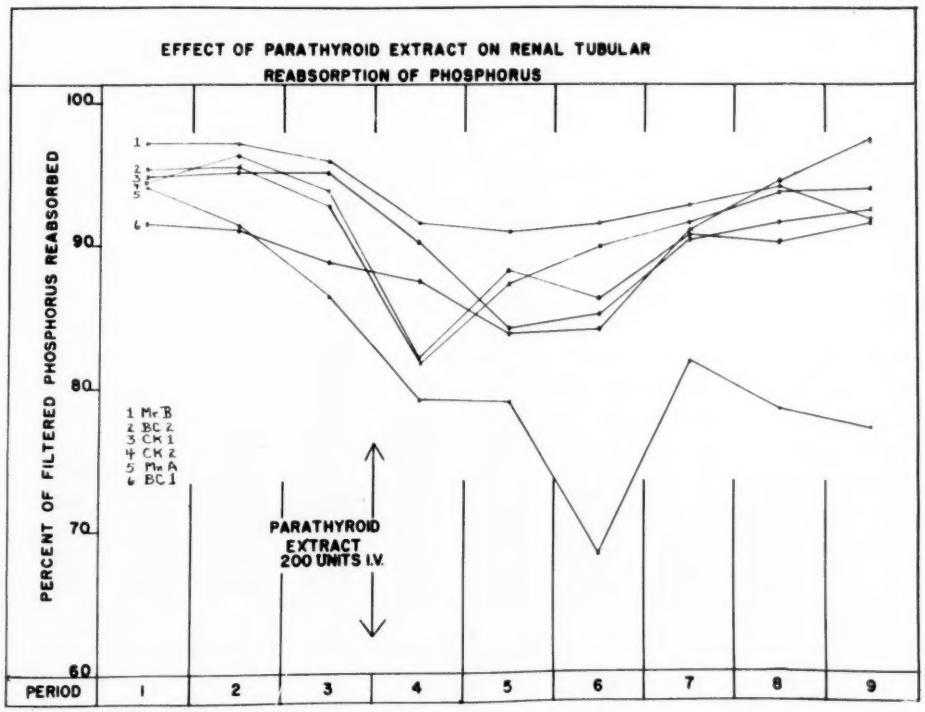

Fig. 1.—Effect of parathyroid extract on renal tubular reabsorption of phosphorus.

ances observed, Table VI. In no experiments was there evidence that a "Tm" for phosphorus existed or had been exceeded.

(e) Urine Flow—Phosphorus Excretion Relationship: In all experiments variations in urine flow were quite marked and showed no correlation with changes in phosphorus excretion associated with parathyroid hormone administration. This is illustrated in Fig. 2 of Experiment 5.

(f) Negative "Rebound" Effect: In two experiments, B. C. (1), Table I, and R. A., Table II, it appeared that after the intravenous hormone effects had "worn off," the tubular reabsorption of filtered phosphorus was greater than dur-

TABLE VI. PHOSPHORUS CLEARANCE ML/MIN. BEFORE AND AFTER PARATHYROID EXTRACT IN NORMAL AND HYPOPARATHYROID INDIVIDUALS

	B. C. (1)	C. K. (1)	B. C. (2)	C. K. (2)	MR. A.	MR. B.	MISS R.	MISS C.
Pre 1	11.49	6.55	7.70	7.34	8.03	4.07	1.60	3.93
Pre 2	12.20	4.86	7.58	4.60	11.03	4.37	1.32	3.94
Pre 3	16.15	6.15	10.08	7.06	15.00	5.70	1.59	4.26
		(200 units of Parathyroid Extract I. V.)						
Post 1	17.60	12.30	25.62	26.40	23.20	8.90	6.77	12.16
Post 2	23.10	19.90	19.90	14.30	24.90	12.40	7.75	23.40
Post 3	23.22	18.61	14.60	17.00	38.30	9.30	9.53	24.80
Post 4	17.60	18.85	11.15	11.48	18.81	12.32	9.96	17.00
Post 5	13.40	12.00	8.95	11.67	21.62	7.60	7.60	13.59
Post 6	8.21	11.60	8.96	9.50	24.30	9.40	6.88	10.00
Post 7	8.20	10.51						
Post 8	4.11	9.53						

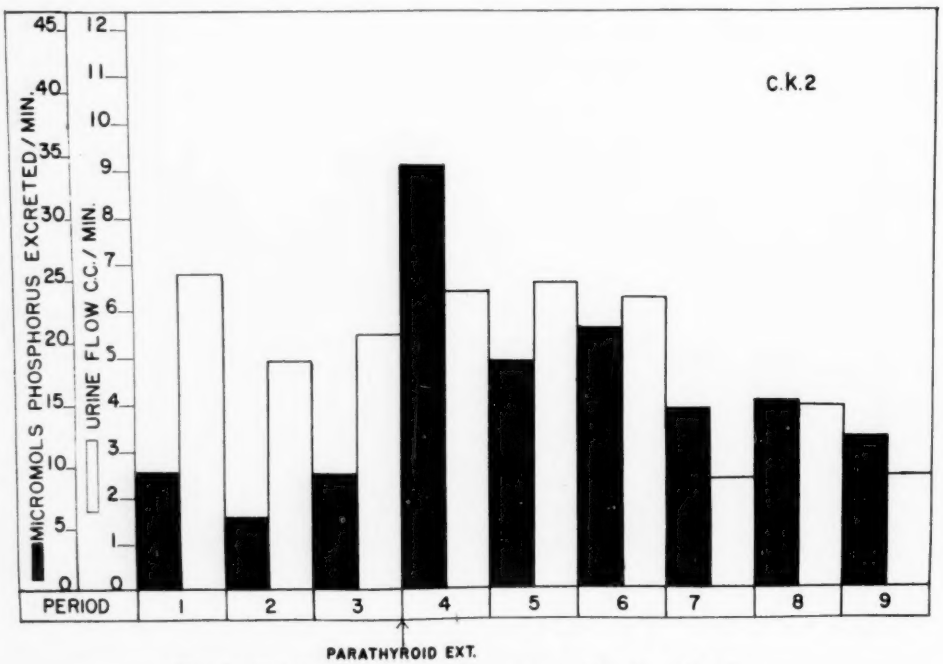

Fig. 2.—Relationship of phosphorus excretion to urine flow.

ing control periods, or, expressed another way, the minute excretion of phosphorus fell below control period levels in both experiments, resulting in phosphorus clearances below the control period levels. In general, the phosphate excretion approached control levels by the fifth postparathyroid hour.

DISCUSSION

Of the following metabolic changes that occur after injection of parathyroid extract—(a) rise in serum calcium, (b) rise in urinary calcium excretion, (c) fall in serum phosphorus, (d) rise in urinary phosphorus excretion—the large rise in minute excretion of phosphorus has been demonstrated in these experiments to be the immediate effect of the hormone. This data is in agreement with the majority of previously reported studies.[1-13, 20, 21, 22, 24, 35]

In the eight- to ten-hour duration of these experiments, this change in phosphorus excretion as noted by others[6, 12, 13] was associated with no significant changes in serum calcium or phosphorus, or urine calcium excretion. That the acute effects of parathyroid hormone are mediated through the kidneys is also amply demonstrated by the experiments of Tweedy, McJunken, and associates[7, 8, 9, 10] in which they studied the effects of parathyroid hormone on radioactive phosphorus distribution, retention and excretion in normal, nephrectomized and thyroparathyroidectomized animals. They noted the immediate increase in radiophosphorus excretion in the normal parathyroid hormone treated animals many times higher than controls. They also noted a redistribution of body phosphorus with an increase in the liver and kidney phosphorus with fall in serum and bone radiophosphorus that lagged behind the phosphorus excretion in the urine. They noted that the parathyroidectomized animals were extremely sensitive to very small doses of the hormone in regard to urine phosphorus excretion. An increased sensitivity was also demonstrated in our two hypoparathyroid subjects as in previous studies on human cases.[1, 6, 12] In Tweedy's nephrectomized animals[7, 8, 9] administration of extremely large doses of parathyroid hormone had no effect on the uptake, retention, distribution or fecal excretion of radioactive phosphorus. This is similar to other studies on nephrectomized animals[2] in which no change in serum calcium occurred after large doses of the hormone were administered. The few experiments[18] that have demonstrated in nephrectomized animals an increase in serum calcium with parathyroid hormone should be critically evaluated in view of the fact that Freeman[16] and others[2, 8] have demonstrated elevations from 1.75 to almost 4 mg. per cent in the serum calcium following simple nephrectomy in animals without administration of parathyroid hormone. Freeman[16] relates this hypercalcemia to elevation in citric acid in the nephrectomized animal. His data did show that parathyroid hormone may have some extra-renal effects in that the citric acid and serum calcium did not rise as high in the nephrectomized and parathyroidectomized animals.

Mention should also be made of the ingenious experiments of Brull[21] in which he demonstrated great increases in phosphorus excretion not only from an animal to whom 100 units of parathyroid hormone had been administered intravenously, but also from a second pair of kidneys irrigated with the first animal's blood.

The failure of parathyroid extract to increase the phosphorus excretion in the children with severe renal disease indicates either that the excretory mechanism for phosphorus is already subject to maximal endogenous stimulation as a result of the elevated serum phosphorus and/or low serum calcium, or that the damaged renal tubules are unable to respond to the hormone. Similar results in renal disease were also seen by Goodby.[13] He showed that after return of renal function in acute nephritics, their ability to respond with increased phosphorus excretion to parathyroid hormone also returned.

The increased phosphorus seen in the present acute experiments, as has been shown in other studies,[3, 11] is not associated with comparable changes in

calcium, nitrogen, or creatinine excretion as would be expected if the immediate increase in phosphorus excretion resulted from breakdown of bone or tissue stores. The constancy of the serum phosphorus level associated with the large increases in phosphorus excretion seen in our experiments could indicate that either the serum phosphorus was stabilized by cellular sources of phosphorus or that the percentage change in extracellular phosphorus was too small to be significantly measured. By roughly calculating the average total extracellular phosphorus in these adults and the amount of phosphorus they excreted under parathyroid hormone stimulation, it appears that significant changes in serum phosphorus should have occurred if no cellular phosphorus had been added to the serum. The present study did not investigate the effects of alteration in the previous dietary intake of calcium and phosphorus on the acute response to parathyroid hormone. This is probably of some significance as these dietary changes may alter the physiologic level of parathyroid activity.*[5, 37, 38, 39, 40]

In the present experiments and others[1, 3] the negative "rebound" or fall in phosphorus clearance and excretion to below control period levels in the final hours after administration of the parathyroid hormone probably merits further study. This phenomenon may partly explain the difference in results seen in other acute experiments when parathyroid hormone is given seven to twenty-four hours prior to the measurement of phosphorus excretion.[15, 17]

The increased phosphorus excretion and clearances after parathyroid extract, Table VI, demonstrated in the present set of experiments could be due to one or more of four possibilities:

(*a*) *An increase in the filterable load of phosphorus due to either increase in serum level or increase in glomerular filtration with no change in the serum level.* Neither of these changes were demonstrated in our experiments after administration of the parathyroid hormone. This is in agreement with the data of Ellsworth and Howard[6] and Cargill and Witham.[20] It is difficult, in view of our findings, to interpret the results in the one case presented by Mitchie and Shorey[23] in abstract form, where the fourfold increase in phosphorus excretion was felt to be due to slight changes in glomerular filtration.

(*b*) *Alteration in the physicochemical state of inorganic phosphate in the serum by parathyroid hormone:* If the hormone were able to increase the filterability of the serum inorganic phosphorus, the product of the serum phosphorus and glomerular filtration rate would not be an accurate measure of filtered load. The increased filtered phosphorus could appear in the urine and lead to an error in the calculated per cent of the filtered phosphorus reabsorbed. We were unable to find references to the effect of parathyroid hormone on the physicochemical state of serum phosphorus. However, Grollman[41] demonstrated that the normal inorganic phosphorus is completely filterable through collodion membranes and Ellsworth and Albright[42] demonstrated that the phosphorus in the serum of one case of hypoparathyroidism was completely diffusible. In view of the foregoing data,[41, 42] alteration in filterability seems unlikely; however, future studies are contemplated to decide this point.

*Since completion of this paper there has appeared in the literature an article by J. D. Crawford, M. M. Osborne, Jr., N. B. Talbot et al.[43] which appears to show the close relationship between phosphorus content of the diet and parathyroid gland activity.

(*c*) *Decreased renal tubular reabsorption of phosphorus:* In the present experiments this appeared to be the primary mechanism accounting for the increased phosphorus excretion. This was readily demonstrated as a marked depression in the per cent of the filtered load reabsorbed and in depression of the reabsorbate concentration, as well as the marked increase in phosphorus excretion and phosphorus clearances.

At first glance it would appear difficult to reconcile the results of Iftakhar and Pitts,[17] Fay and co-workers[15] and Hogben and Bollman,[14] with those of the present study and others.[6, 20] The former workers[14, 15, 17] felt that parathyroid hormone had no effect on the tubular reabsorption of phosphorus. However, certain major important differences in the experiments exist. In all three of these studies[14, 15, 17] phosphorus excretion was measured during and after large intravenous phosphorus infusions that increased plasma levels as much as fifteenfold. At these high levels there is some question as to a change in the filterability of the serum phosphorus.[24, 28] However, some studies[33, 34, 36] show that it is probably all filterable. Of even more importance is the probable difference in the mechanisms of excretion of endogenous and exogenous phosphates. Ollayos and Winkler[33] demonstrated that at normal endogenous levels throughout a twenty-four hour period the concentration of phosphorus in the serum did not determine the rate of excretion of phosphorus and large variations in phosphorus excretion occurred without change in serum level. This is in marked contrast to rates of excretion with rising plasma levels. They, as well as others,[15, 19, 36] demonstrated progressive increases in the clearance of phosphorus with progressive increases in the serum level. In addition, in contrast to Harrison and Harrison[24] and Ayer, Schiess, and Pitts,[25] Smith, Ollayas, and Winkler[34] were unable at any time to demonstrate a "Tm," although their serum concentrations reached levels much higher than the above experiments[24, 25] and reabsorptive values reached three to four times the supposed maximum. Therefore, the increased clearance with rising plasma levels cannot be explained by exceeding "Tm," but must be due to alteration of the kidney's response at high serum concentrations, possibly an increase in tubular secretion of phosphorus. Under these circumstances the effect of parathyroid hormone on renal excretion and tubular reabsorption of phosphorus may be completely masked by the high clearances already attained in the control periods.

In the presence of a rising clearance with rising plasma levels, and in the absence of a definite "Tm," it appears that at high serum concentrations we are dealing with an excretory mechanism closely dependent on serum concentration and at endogenous levels we are dealing with a mechanism relatively independent of serum levels. To point out even further this difference in endogenous and exogenous excretion, the studies of Govaerts[36] with infusions of radioactive phosphorus demonstrated that injected phosphate in pure inorganic form is excreted or cleared by the kidney at a much faster rate than endogenous phosphate, i.e., the exogenous phosphate making up a much larger percentage of the excreted phosphorus than of the serum phosphorus. This phenomenon was noted even though the infusions of radiophosphorus did not elevate the serum phosphorus levels.*

From the above discussion it can be seen that study of the effects of parathyroid hormone on excretion of high levels of infused phosphorus[14, 15, 17] cannot be compared closely with studies of the effect of parathyroid hormone on endogenous phosphorus excretion. It should also be pointed out that the increase in phosphorus excretion under physiologic conditions, including parathyroid stimulation, takes place without any evidence for the saturation of a reabsorptive capacity, if such even exists. In the previous studies of Fay and co-workers[15] and Iftakhar and Pitts[17] renal phosphorus excretion was not examined until four and one-half to twenty hours after administration of the hormone (a point at which a "rebound phenomenon" may well be acting).

(*d*) *Tubular secretion of phosphorus:* The term "net" tubular reabsorption of phosphorus was used in expressing the results of the present study because the calculated percentage decrease in tubular reabsorption of phosphorus after parathyroid hormone may actually have been due to tubular secretion with little or no change in the amount reabsorbed, the "net" reabsorption being the result of both secretion and reabsorption. That tubular secretion of phosphorus may take place is suggested by the work of Barclay and associates[29, 30, 31, 32] with phosphate infusions in both dogs and human beings, and by endogenous phosphate clearance exceeding inulin clearance in one patient in Ingbar and co-workers'[27] ACTH studies. It is also suggested in the studies of Rappaport and associates[43] where they demonstrated the very similar excretory responses of phosphates and potassium to osmotic diuresis. Potassium is now known to have a tubular secretory component.

It is well known that phloridzin is an inhibitor of phosphorylation processes and probably inhibits active enzyme systems. In regard to the renal tubule, it might be considered analogous to mercury in its action toward potassium, sodium and para-aminohippurate, etc. Pitts[19] demonstrated that phloridzin produced a definite fall in the rising phosphate clearances associated with rising plasma levels; while in slightly later experiments Walker and Hudson[26] showed that in the amphibious nephron phloridzin definitely inhibited the tubular reabsorption of phosphorus. If we assume that both these results are due to an alteration in the tubular reabsorption of phosphorus, we must then postulate that in the first case phloridzin increased or stimulated the tubular reabsorption of phosphorus and in the latter it inhibited this same process. It is more reasonable to postulate that phloridzin acted as an inhibitor in both experiments. In Pitts' loading experiments[19] it inhibited tubular secretion and in the latter,[26] where primarily reabsorption was taking place, this also was inhibited. This blockage of both an active secretory and reabsorptive process has been demonstrated by Mudge and associates[44] for mercury's action on potassium excretion.

In the physiologic state with normal phosphorus levels in the serum almost all the filtered phosphorus is reabsorbed. This was seen in the control periods of the present experiments. After phosphate infusion or after parathyroid stimulation, tubular secretion of phosphorus may be very significant. The possibility is not remote, as stated by Mudge and co-workers[44] in regard to potassium, that un-

*Since submitting this manuscript for publication, Handler and Cohn's[37] data have appeared in the literature. Although they obtained experimental results similar to Govaerts,[36] they seriously question his interpretation.

der normal circumstances a major portion of the phosphorus excreted in the urine is largely defined by the magnitude of the secretory process. If a clearance of phosphorus above that of glomerular filtration could be demonstrated after parathyroid hormone administration and the clearance decreased by phloridzin, the above hypothesis would be strengthened. Further studies are contemplated to investigate the problem of tubular secretion of phosphorus with parathyroid stimulation.

The changes in electrolyte excretion other than phosphorus, and the changes in pH, ammonia, and titratable acid are very variable, both in this and other studies.[11, 12, 35] It would appear that a greater number and more controlled studies in regard to previous dietary intakes of sodium, potassium, chloride, etc., should be done before any conclusions can be drawn.

SUMMARY AND CONCLUSIONS

1. The present study confirms the results of previous work showing that the injection of parathyroid extract causes an immediate increase in urinary phosphorus excretion.

2. The mechanism involved appears to be a decrease in the percentage reabsorption of the filtered phosphorus; however, the possibility that parathyroid hormone stimulated the tubular secretion of phosphorus is suggested.

3. These results do not necessarily indicate that parathyroid hormone has only one site of action but only that the acute effects are mediated through the kidneys.

4. The importance of distinguishing between experiments done on exogenous and endogenous phosphate excretion is stressed, as well as the importance of time in the interpretation of parathyroid hormone effects.

5. No acute effect of parathyroid hormone was noted in the presence of chronic renal disease.

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A CRITICAL EVALUATION OF THE TOXICITY OF PEMPHIGUS SERUM (PELS-MACHT TEST)

G. Douglas Baldridge, M.D.,* and Albert M. Kligman, M.D., Ph.D.
Philadelphia, Pa.

THE cause of pemphigus is unknown. The disease often has been considered to be "toxic" in origin.[1] A test has been proposed by Pels and Macht which presumably detects a "toxin" in the serum of patients with this disease.[2] The procedure depends upon the inhibition of growth of *Lupinus albus* seedlings when immersed in a 1 per cent solution of blood serum. The method is generally known as the phytopharmacologic test for pemphigus.

The test as represented by the original authors is not specific for pemphigus. Toxins with a similar phytopharmacologic effect are said to be demonstrable sometimes in patients with paresis[3] and pernicious anemia[4] and in normal menstruating women.[5] A multitude of papers has been published by Macht in which the diagnostic virtues of the test are detailed.[6]

There has been considerable discussion among dermatologists as to the significance or dependability of the phytopharmacologic test for pemphigus. Serious experimental evaluation is, however, lacking, except for the work of Greb and Hollander who in a short series of cases were unable to confirm Macht's findings.[7]

The present study has the objective of evaluating the presence of toxic factors in the serum of patients with pemphigus. Three biologic test materials were employed:

(1) The inhibition of growth of *L. albus* seedlings (Pels-Macht test).

(2) The inhibition of fibroblast proliferation and leucocyte migration in tissue culture.

(3) The inhibition of germination of the macroconidia of *Microsporum fulvum,* one of the ringworm organisms.

METHOD AND MATERIALS

The test sera were obtained from six patients with undoubted active pemphigus. The sera were immediately separated by centrifugation and if not used immediately were stored at –20° C. As soon as possible (within two days), these sera were studied as follows.

(1) *Inhibition of Growth of the Seedling L. albus (Pels-Macht Test).*—Except for the fact that normal human serum controls were run concurrently with the pemphigus sera, the tests were set up exactly as described in the publications of Macht and associates. Briefly,

From the Department of Dermatology and Syphilology, School of Medicine, University of Pennsylvania (Donald M. Pillsbury, M.D., Director).

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*Present address: Department of Medicine, University of California at Los Angeles.

the seedlings of *L. albus* were germinated in tap water and then allowed to develop further in damp Sphagnum moss. After making the base line measurements of root length, they were placed rootling down in the appropriate test solution. Both the pemphigus sera and the normal control sera were diluted to 1 per cent with Shive's nutrient solution.[8] After twenty-four hours' incubation at room temperature the rootlings were again measured and the average increment of seven to ten seedlings was taken as the experimental result.

(2) *Tissue cultures of Chick Embryo Fibroblasts and Human Buffy Coat Leucocytes.*—The slide culture technique was used.[9] Fibroblast material was obtained from either the heart or thigh muscle of the developing 8-day-old chick embryo. The tissues were placed immediately in a Petri dish containing Tyrode's solution, cut into pieces 1 mm. square, and explanted to the cover slip. One drop of chick plasma was placed on the explant followed by 1 drop of undiluted pemphigus serum. Normal serum was used in the control preparations. Finally, a drop of 25 per cent chick embryo extract in Tyrode's solution was added to produce a clot. The cultures were placed in the 37° C. incubator immediately after they were prepared. Similar preparations were made employing human buffy coats (leucocytes) as the explants. With these the effect of pemphigus and normal sera on cellular migration was studied. The tissue cultures were examined for inhibition of growth or migration at twenty-four hour intervals.

(3) *Germination of Microsporum fulvum Macroconidia.*—Suspensions of *M. fulvum* macroconidia were prepared in normal saline so as to contain five to ten macroconidia per high-power field. These spores were collected from the surface of a fourteen-day culture of the fungus on Sabouraud's medium. To perform the test, 0.05 ml. of the suspension was added to 0.05 ml. of the test serum on a glass slide maintained in a Petri dish moist chamber as described in a previous report.[10] A serum concentration of 50 per cent was thus attained. The effect of the sera on germination was measured by counting 100 consecutive spores after thirty hours of incubation and recording the percentage of these showing one or more germ tubes. These tests were run in parallel fashion using five normal human sera for controls.

RESULTS

(1) *Inhibition of Growth of L. albus Seedlings.*—Examination of Table I shows that under the conditions of this experiment there was no inhibition of rootling growth by 1 per cent pemphigus sera as compared with the controls.

(2) *Inhibition of Tissue Cultures.*—The results are tabulated in Table II. It is apparent that as compared with the controls, pemphigus sera have no properties toxic to either embryonic fibroblasts or human buffy coat leucocytes.

(3) *Germination of M. fulvum Spores.*—The results recorded in Table III demonstrated quite clearly that under the conditions of this test the germination of spores was not inhibited by the presence of pemphigus sera.

TABLE I. INHIBITION OF GROWTH OF L. ALBUS SEEDLINGS

PATIENT	AV. INCREMENT IN ROOT LENGTH 1% PEMPHIGUS SERA (24 HR.)	AV. INCREMENT IN ROOT LENGTH. 1% NORMAL CONTROL SERA (24 HR.)
1	12 mm.	9 mm.
2	10 mm.	13 mm.
3	6 mm.	4 mm.
4	8 mm.	7 mm.
5	13 mm.	12 mm.
6	9 mm.	9 mm.
Average	9.6 mm.	9.0 mm.
Standard deviation	2.4	3

TABLE II. INHIBITION OF TISSUE CULTURE GROWTH

PATIENT	EXPLANT	T.C.	AV. GROWTH IN PEMPHIGUS SERA	T.C.	AV. GROWTH IN CONTROL NORMAL SERA
1	Fibroblast	8	+++*	4	+++
	Buffy coat	3	+	2	++
2	Fibroblast	6	++	4	+++
	Buffy coat	3	++	2	++
3	Fibroblast	4	+++	4	+++
	Buffy coat	4	+++	4	+++
4	Fibroblast	3	++	3	++
	Buffy coat	3	+++	3	++
5	Fibroblast	3	+	3	++
	Buffy coat	3	++	3	++
6	Fibroblast	3	++	3	++
	Buffy coat	3	+++	3	++

*The designation "+++" indicates a proliferation of growing cells three-fourths the distance across a low-power microscopic field; + indicates a spread of one-fourth the distance, etc.

TABLE III. INHIBITION OF GERMINATION OF M. FULVUM SPORES

SERUM	PERCENTAGE GERMINATION			SERUM	PERCENTAGE GERMINATION		
	RUN 1	RUN 2	AVERAGE		RUN 1	RUN 2	AVERAGE
Pemphigus 1	86	84	85	Normal 1	84	91	87.5
Pemphigus 2	93	77	85	Normal 2	76	83	79.5
Pemphigus 3	81	87	84	Normal 3	90	88	89.0
Pemphigus 4	79	73	76	Normal 4	78	63	70.5
Pemphigus 5	80	73	76.5	Normal 5	71	77	74
Pemphigus 6	90	82	86				
Mean			82.1				80.1
Standard dev.			4.2				7.3

DISCUSSION

Cells in tissue culture are notoriously sensitive to toxic agents.[11] Traces of impurities in distilled water have sometimes accounted for the failure of the explants to grow. The tissue culture method has the advantage over animal experimentation in permitting a relatively pure type of preparation in which the variables which could affect growth are easily controlled. Pemphigus is a fatal disease with widespread cutaneous manifestations. If these latter result from circulating "toxins" it is improbable that cells in tissue culture would remain unaffected by these same toxins. Macht has, however, pointed out that agents which are not toxic to animals may be toxic to plants.[12] The studies reported herein with *L. albus* seedlings and with *M. fulvum* spores fail to confirm the hypothesis of a plant toxin in the serum of pemphigus patients. Phytopathologists often use the inhibition of fungus spore germination as a test for the presence of clinical toxins.[13] The standard LD_{50} method is based on this procedure.

The support for the theory of "toxicity" as the etiologic basis for pemphigus has depended upon the Pels-Macht procedure. The details of the procedure are therefore worthy of closer scrutiny. It is odd that so well known a test has gone unconfirmed for twenty years except by the original presenter. One possible reason for this is the presence in many of Macht's papers of stern warnings such as the following, ". . . . you must be careful in learning the technique.

You will probably fail on the first, second or the first half dozen attempts'' and, ''one has to be trained in animal pharmacology and plant physiology'' (to perform the test)[14] and again, ''In this highly specialized work as in any complicated laboratory technique, negative results in the hands of a tyro count for little or nothing.''[15] These warnings are remarkable in view of the simplicity of the test in reasonably competent hands. The difficulties emphasized by Macht have not been apparent in this study.

Certain inadequacies are apparent in the standards set up by Macht. A ''standard'' twenty-four hour growth increment for the seedlings was established by growing them in a mixture of Shive's nutrient solution and distilled water. These results were taken as 100 per cent or ''normal'' growth. When the seedlings were grown in 1 per cent normal serum in Shive's solution, it was found that the growth rate was about 70 to 75 per cent of that in Shive's solution mixture. Normal sera thus evidently depress the growth rate as compared with a nutrient solution. Sera having a growth rate less than 70 per cent were arbitrarily considered abnormal. Macht found that pemphigus sera averaged about 55 per cent. These are, of course, inadequate controls, since the ''control'' sera are not being evaluated at the same time and under the same conditions as the test sera. Moreover, the standard of reference is not normal serum but a nutrient solution. This has the effect of emphasizing the supposed inhibition found with pemphigus serum. If the inhibitory effects of pemphigus sera are compared with the inhibitory effects of normal sera, the differences found in Macht's figures are necessarily reduced. Any number of extraneous factors could operate to vitiate the results obtained by a crude biologic method in which the controls are not tested simultaneously with the suspected sera. This was avoided in the present study. This difference in the nature of the controls possibly accounts for the data presented herein in which there was no evidence in support of Macht's findings. There are other difficulties with the test. For example, in a recent summation the authors state that ''occasional crops of *L. albus* yield seedlings impervious to saline solutions or toxins. Such seedlings give a negative reading or an index considerably higher than yielded by controls in normal serum.''[15] It is not stated how results from these experiments are considered but by implication one must assume that they are disregarded in calculating results. Again to quote: ''In dealing with specimens of serum of patients suspected of having pemphigus, pernicious anemia or some similar condition, negative or exceedingly high readings after a single examination should be regarded as 'no takes' if in several repetitions of the test, toxic effects are definitely established.''[15] This remarkably compromising statement allows, consciously or unconsciously, the prejudices of the investigator to become a factor in deciding what results are acceptable. With a means such as this at hand to permit rejection of ''bad'' results, one is highly favored with the opportunity to secure ''good'' results. Calculation of results on these terms will nearly always result in ''significant'' data.

In an extensive study on human sera from patients with various diseases including pemphigus, Ball has found a complete absence of inhibition of spore

germination in the plant *Sclerotina fructicola.*[16] This author also repeated the Pels-Macht procedure and produced results which coincide completely with the work reported here.

CONCLUSIONS

Tissue culture studies of chick embryo fibroblasts and human buffy coat show no evidence of a toxic fraction in pemphigus sera.

Germination of the spores of *M. fulvum* was not prevented by pemphigus sera.

The results reported by Pels and Macht with pemphigus could not be confirmed.

Under the conditions of this experiment, no evidence to support the theory of a toxic factor in the etiology of pemphigus could be demonstrated.

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THE EFFECT OF CORTISONE ON TUBERCULOSIS IN THE GUINEA PIG

ROBERT G. BLOCH, M.D., KIRSTEN VENNESLAND, M.D., AND CLIFFORD GURNEY, B.S.
CHICAGO, ILL.

THE effect of cortisone on experimental tuberculosis has been studied by several investigators who reported a number of changes in the early manifestations of tuberculous infection and the later development of the disease process. The concept of the general depressive effect of the drug on hypersensitive states induced by infectious agents, accepted by most authors, has been studied for the specific tuberculous infection through the medium of tuberculin tests by a few investigators. The depressive effect has been acknowledged by a number of authors, although there is a difference of opinion as to the amount of cortisone which is required to achieve it.[1, 2] No reports on the changes of the blood sedimentation rate in experimental tuberculosis in the guinea pig seem to be available. The tissue changes in tuberculosis have been reported as being different in cortisone-treated animals from those in untreated controls.[3, 4] The effect of streptomycin therapy has been described to be altered when the treatment is combined with the administration of cortisone.[3]

The experiments which are presented in this paper were aimed at a comprehensive view of the effect of cortisone on the tuberculous guinea pig. They include some studies on the response of endocrine glands in an attempt to find an opening into their possible role in tuberculous infection; their participation in the response to tuberculosis was suggested long ago.[8, 9, 13] Cortisone was given over longer periods of time than in the experiments so far reported in order to study its effect on prolonged administration. In addition, the response of the cortisone-treated guinea pig to discontinuation of extended treatment was of interest.

MATERIALS AND METHODS

One hundred twenty-four guinea pigs weighing between 400 and 500 grams were inoculated in the right groin with 0.1 mg. of human strain of *mycobacterium tuberculosis* H37Rv, which had been grown in Proskauer-Beck medium and which was then ground in a sterile agate mortar and suspended in Dubos medium. One hundred two of the animals were selected for treatment with streptomycin, cortisone, and the combination of the two drugs. They were divided into two main groups. (See Table I for tabulation of experiment arrangement.) In Group I treatment was begun on the day of inoculation with tubercle bacilli. In Group II the injections were started twenty-four days after inoculation, at which time autopsy findings in pretreatment controls showed tuberculous disease to have progressed. Each of the two groups was again divided into two subgroups, one of which was

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killed at the termination of treatment of twenty-seven days' duration (Groups IR and IIR), while in the other subgroup the animals were permitted to survive the termination of treatment (Groups IS and IIS). The survival period in Group IS was fifty-five days from the termination of treatment and eighty-two days from inoculation; in Group IIS it was thirty-six days from the termination of treatment and eighty-seven days from inoculation. The survival period of both groups from the day of inoculation was thus equalized to within the five-day period which was required to complete the necropsies. All of the groups were subdivided for treatment with streptomycin (A), cortisone (B), and the combination of streptomycin and cortisone simultaneously (C). The same dosage of each drug was used in Group C as in Groups A and B. Twenty-two animals served as controls; a part of the control group was killed at the termination of the experiment in each of the treated groups. All animals were weighed at weekly intervals.

TABLE I. ARRANGEMENT OF EXPERIMENT AND TUBERCULOSIS INDEX

	GROUP I				GROUP II					
	27 DAYS OF TREATMENT BEGUN ON DAY OF INOCULATION				27 DAYS OF TREATMENT BEGUN 24 DAYS AFTER INOCULATION					
	IR KILLED AT END OF TREATMENT		IS 55-DAY SURVIVAL		PRETREATMENT		IIR KILLED AT END OF TREATMENT		IIS 36-DAY SURVIVAL	
TREATMENT										
Untreated controls	6.5	(8)	9.3	(4)	6.5	(8)	8.3	(9)	9.3	(4)
A. Streptomycin 8 mg./day	2.1	(7)	6.8	(8)			5.9	(8)	7.0	(6)
B. Cortisone 5-10 mg./day	7.4	(14)	9.3	(4)			9.6	(9)	10.2	(5)
C. Streptomycin and cortisone	4.0	(8)	8.6	(9)			4.0	(9)	5.5	(7)

The tuberculous index is calculated from macroscopic findings comprising tuberculous involvement of the lung, liver, spleen, and kidneys, grading each organ from 1 to 4. The macroscopic findings are checked by histologic examination for the specificity of involvement and for the presence of lesions not discovered on macroscopic examination. The maximum index possible is 16. The figures given are the average indices for each group. The figures in parentheses are the number of animals in the groups.

Streptomycin was injected subcutaneously in one daily dose of 8 mg.; the cortisone injections were made intramuscularly in two equal daily doses. In Groups IRB and ISB the daily cortisone dose was 10 mg. for an initial period of five days. It was then graded down to 5 mg. daily for a period of eight days, followed by an increase to 10 mg. for seven days in order to observe the effect of the larger dose on the tuberculin reaction. For the remainder of the treatment period the 5 mg. daily dose was used. Groups IIRB and IISB received 5 mg. cortisone daily throughout the treatment period. This essential daily dose of 5 mg. was chosen on the basis of the experience of other investigators that the cortisone requirement per body weight of the guinea pig for the purpose of achieving noticeable effect is greater than that of human beings. The dosage employed by several authors varied between 5 and 20 milligrams.[1, 2] We felt at the beginning of the experiment that a minimum of 5 mg. daily should be the dose of choice; however, in order to make certain that this would not be unnecessarily large, an accessory experiment was carried out employing smaller doses. The arrangement of this experiment as to infection with tuberculosis and period of treatment was identical with that described for the main experiment. Three groups of four guinea pigs each received injections of 1, 2, and 3 mg. daily in two equal doses. The effectiveness of these different doses was ultimately appraised chiefly by their effect on the adrenal glands as the best means to determine cortisone effect anatomically. The results were compared with the 5 mg. group and the controls in the larger experiment.

Tuberculin tests with 0.01 Gm. of old tuberculin intracutaneously were carried out in all animals in Groups IR and IS fourteen and twenty days after the beginning of treat-

ment; in the survivor Group IS they were repeated six, twenty, and thirty-seven days after treatment had been discontinued. In Groups IIR and IIS the tests were made before treatment, on the seventh and twenty-fifth day of the treatment period, and were repeated fifteen days after treatment had been discontinued. Readings of the reactions were made after twenty-four and forty-eight hours, and the forty-eight hour value was recorded on an arbitrary scale of 0 to 4 plus for both peripheral wheals and necrotic centers.

The blood sedimentation rate (30- and 60-minute values) was determined in all animals before inoculation and the determinations were continued at intervals of from seven to ten days until termination of the experiment. Eosinophile counts were made in five to six animals: in the animals of all Groups I four times at intervals of seven days; in all Groups II before and after treatment as well as twice during the treatment period.

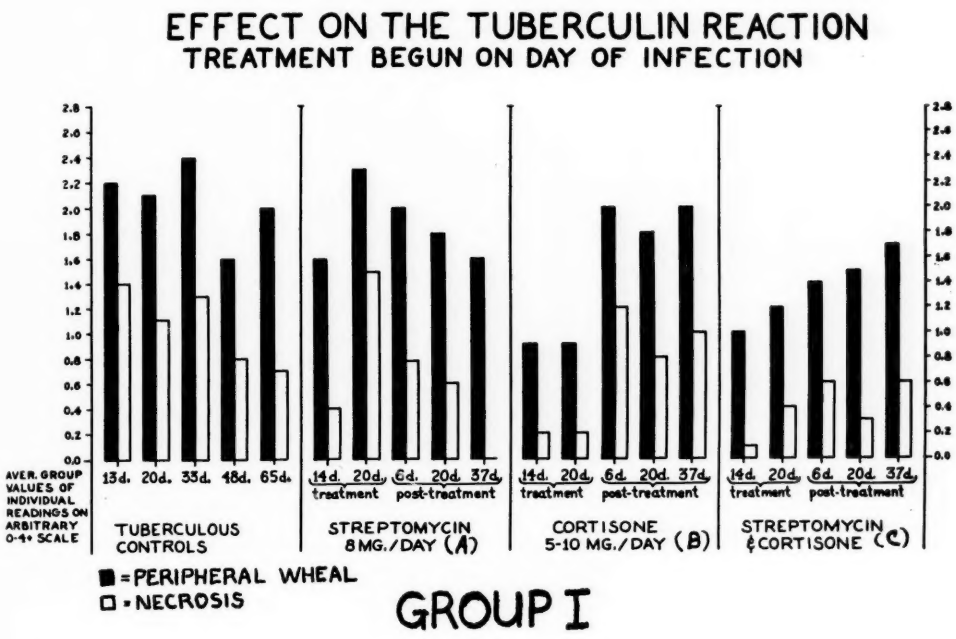
EFFECT ON THE TUBERCULIN REACTION
TREATMENT BEGUN ON DAY OF INFECTION
AVER. GROUP VALUES OF INDIVIDUAL READINGS ON ARBITRARY 0-4+ SCALE
13d. 20d. 33d. 48d. 65d.
TUBERCULOUS CONTROLS
14d. 20d. treatment
6d. 20d. 37d. post-treatment
STREPTOMYCIN 8 MG./DAY (A)
CORTISONE 5-10 MG./DAY (B)
STREPTOMYCIN & CORTISONE (C)
■ = PERIPHERAL WHEAL
□ = NECROSIS
GROUP I

Fig. 1.

The animals were killed by ether and complete autopsies were performed on all of them. The tissues were fixed in Formalin and sections stained with hematoxylin-eosin, as well as by the Ziehl-Neelsen stain, for demonstration of acid-fast bacilli. The weight of the thyroid and adrenal glands was determined in the survivor Group IS and in all animals of Group II. Fat stains, using scarlet red, were made of sections of the adrenal glands of Group IS and all of Group II. The pituitary gland was removed from the animals in the survivor Group IS and from all the animals in Group II, exclusive of those which died spontaneously. The weight of the gland was determined in some of the animals of Group II, in some infected controls, as well as in a few uninfected cortisone-treated and untreated pigs. This practice was discontinued when the average weight of the gland in all groups proved to be relatively constant, varying between 17.1 and 19.1 milligrams.

RESULTS AND DISCUSSION

Clinical Observation During the Experiment.—From the beginning of the cortisone injections, the animals in that group showed signs of illness and loss

of weight. The early mortality in cortisone-treated Group IB was higher than in the other groups. Death in all cases was due to intercurrent nonspecific infection, most frequently pneumonia. In a few of these animals massive unilateral or even bilateral hemorrhagic pleural effusions were the immediate cause of death. The mortality decreased when the initial daily cortisone dose of 10 mg. was reduced to 5 mg., but throughout the experiment the pigs in this group were the most listless and showed the least resistance to disease. Those which survived to the termination of the experiment were at the time more emaciated than the animals in any other group, including the controls.

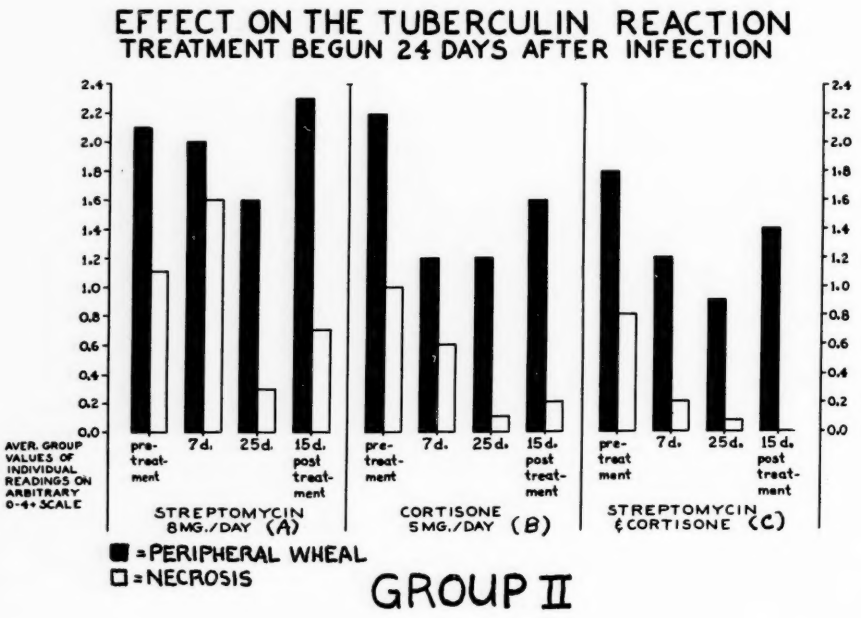

Fig. 2.

The intra-vitam effect of treatment was followed essentially through observations on tuberculin sensitivity and variations of the blood sedimentation rate and blood eosinophile counts.

Tuberculin Sensitivity.—The results of repeated tuberculin tests in all of the groups are recorded in Figs. 1 and 2. It seemed to us that the frequency and intensity of necrotic centers was as good, if not better, a criterion of response as the size and degree of redness of the peripheral wheal. The variations in the streptomycin-treated group (A) were not of significant magnitude, especially in view of the often-observed spontaneous fading of sensitivity after its first rise following infection. The inhibitory effect of cortisone (B) on the reactions in both Groups I and II was approximately 50 per cent of the average value when judged by the peripheral wheal and even more striking when measured by the average effect on the occurrence of necrosis, which in Group

II (Fig. 2) was only about one-tenth of the initial value after twenty-five days of treatment. In both Groups I and II, the sensitivity rose or returned, respectively, to values comparable to those in the control animals as soon as treatment was discontinued. These findings are similar to those of others who reported partial suppression of specific hypersensitivity in patients but failure of the attempt to inhibit tuberculin response completely.[5] The opinions as to the daily dose of cortisone required for even partial inhibition in guinea pigs seem to differ.[6] Harris and Harris[1] used doses of over 10 milligrams. This, in our experience, is excessive. In fact, there seemed to be little difference in our experiment between the inhibitory effect of the 5 mg. and 10 mg. doses. The variations of hypersensitivity under cortisone therapy combined with streptomycin (C) were essentially the same as with cortisone alone.

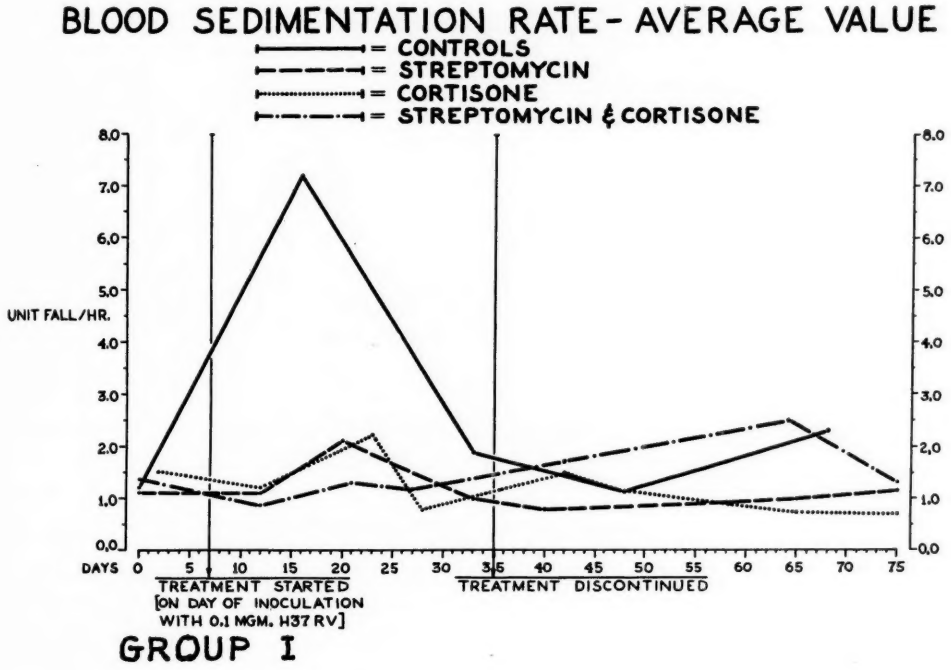

Fig. 3.

Blood Sedimentation Rate.—The average values of the blood sedimentation rate in the control animals had risen sharply ten days after inoculation, while in the treated pigs of Group I they remained unchanged. Fig. 3 shows that this occurred not only in the cortisone-treated animals but also in those under treatment with streptomycin alone. It signifies that two wholly different forces were at work—the inhibitory effect of cortisone on body response and the bacteriostatic effect of streptomycin. Either of these influences alone was strong enough to induce nearly complete suppression of the sedimentation rate. The values in the control group had returned to the initial level after about thirty

days, thus presenting the rise of the blood sedimentation rate in the tuberculous guinea pig as an initial and temporary phenomenon. In the groups in which treatment was begun twenty-four days after inoculation (Fig. 4) the same average initial rise was observed in all animals. The return to pretreatment values in the treated animals concurred with that in the controls, thus precluding any interpretation as to the possible effect of the drugs and their combination on this phenomenon.

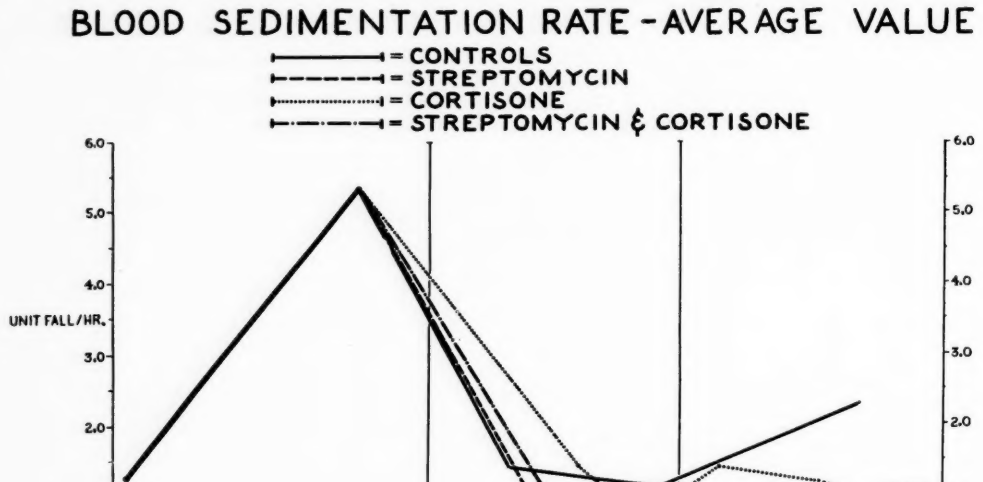

Fig. 4.

Eosinophile Blood Cells.—The depressive effect of cortisone on the eosinophile count in normal guinea pigs has been reported by others.[7] We found that the number of eosinophile cells in the normal guinea pig's blood varies widely. In a group of nine healthy animals the values ranged from 6 to over 1,800. However, they never were entirely absent. Therefore, treatment could not be assumed to have affected the count unless no eosinophile cells were present at all. In groups B and C (treated with cortisone and cortisone plus streptomycin) eosinophile cells were absent in the blood of eight out of twelve animals and in nine out of eleven of the same group in a second count. In the remaining two animals the count was very low at 11 each. The average count in the group treated with streptomycin alone did not differ from the results in the control group. Cortisone, therefore, has a definite suppressive effect on the eosinophile count in the blood of the tuberculous guinea pig.

Pathologic Findings.—The pathologic findings as appraised by the tuberculosis index are recorded in Table I. Streptomycin strongly inhibited the early

development of disease in the immediately treated Group IA, but only temporarily so. There was comparatively little tuberculosis in Subgroup IRA, killed at the end of treatment, but three times as much in Group ISA after a survival period of fifty-five days. In the pigs receiving delayed treatment (Group II) the results were practically identical in both Subgroups R and S. Tuberculosis in animals receiving streptomycin in this delayed treatment group (Group IIA) was definitely less extensive than in the controls, having been held at the pretreatment level. The poor physical condition of most of the cortisone-treated animals, especially early in the experiment, and their high initial mortality has been mentioned already. However, the number of spontaneous

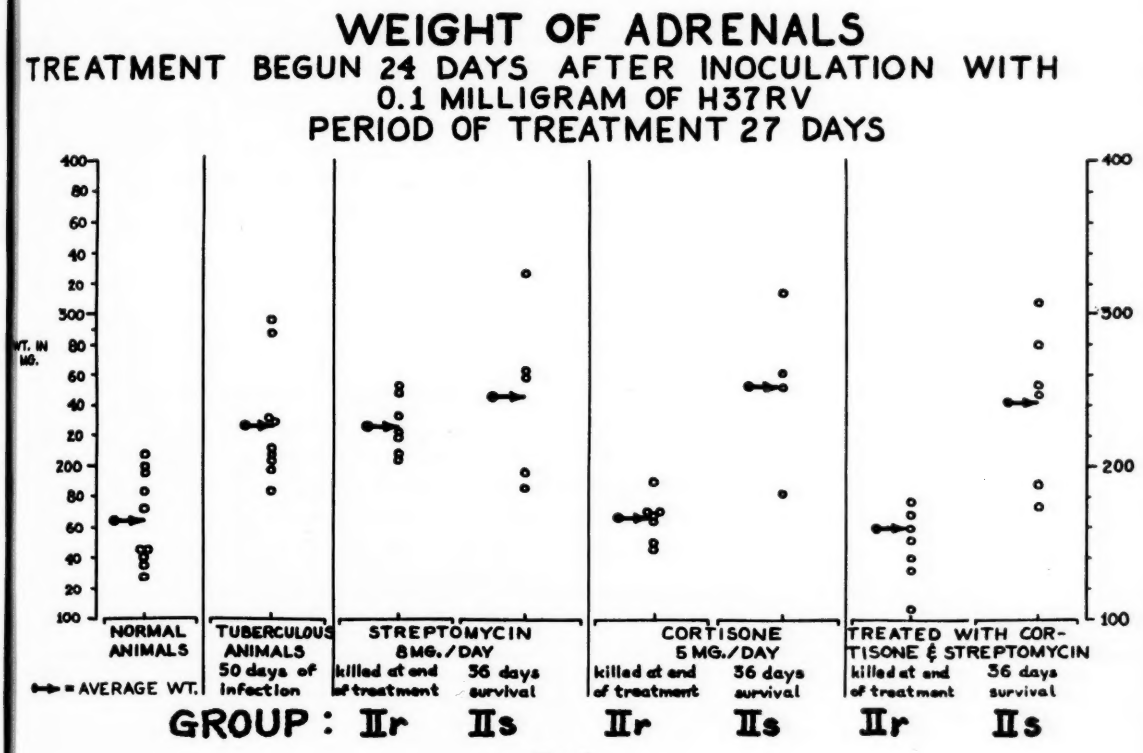

Fig. 5.

deaths decreased as the experiment progressed, and the extent of the average tuberculous involvement in both Groups IB and IIB at the end did not exceed that in the control animals. In Group IB it was equal to that in the controls. The guinea pigs treated with a combination of streptomycin and cortisone from the day of infection (Group IC) had more tuberculosis than those treated on the same schedule with streptomycin alone. But when the combined treatment was begun twenty-four days after inoculation (Group IIC), the animals did not have more but rather somewhat less disease than the corresponding streptomycin-treated group and considerably less than the controls. These findings indicate that cortisone promotes the development of tuberculosis in the

guinea pig, especially during the early stage of infection, and apparently particularly so during the first rise of hypersensitivity. When cortisone was given together with streptomycin therapy, the predominance of early damage was strikingly evident, while the beginning of treatment after the development of hypersensitivity did not seem to impair the effect of streptomycin.

The macroscopic and histologic appearance of the tuberculous lesions did not impress us as sufficiently different in the various groups to conclude that cortisone had exercised a marked influence on the nature of tissue response. Spain and Molumut[3] reported more extensive and less localized tuberculous lesions in cortisone-treated guinea pigs than in control animals. Lurie and coworkers[4] in a preliminary paper reported the development of greater numbers of tubercles in the lungs of cortisone-treated rabbits after infection by inhalation than in untreated controls. They described definite differences in the histologic appearance of the tuberculous lesions and in the number of acid-fast bacilli present in them. Possibly the different mode of inoculation, the shorter period of their experiment, and the lesser extent of tuberculous changes, especially in internal organs, could acount for differences which we cannot substantiate from our own findings.

The examination for acid-fast bacilli in tissue sections stained by the Ziehl-Neelsen method revealed that in the untreated control group, killed twenty-seven days after infection, organisms were rather easy to find in most of the animals. As the survival time of the animals increased, organisms became increasingly difficult to locate. As would be expected from both the character and extent of the lesions in the groups receiving streptomycin, bacilli were extremely hard to find in the organs of these animals, especially in those which were treated from the beginning of infection. This was true in both the groups killed immediately after treatment and in the survival group. Animals receiving cortisone from the time of infection and killed at the end of the treatment period had lesions which were almost uniformly swarming with bacilli. In those treated with cortisone starting twenty-four days after infection and killed at the end of the treatment period, bacilli were present but few in number, not differing much from the control group. However, the six animals of this group which died spontaneously from two to twenty-six days after the beginning of treatment showed enormous numbers of bacilli in the lesions. Four of these were not considered in the tuberculosis index for the group because the duration of treatment was too short.

As yet nothing is known about a possible influence of cortisone on the tubercle bacillus per se. Growth experiments with cortisone are in progress in our laboratory.

Adrenal Glands.—The size and weight, as well as the anatomic pattern of the adrenal glands showed some significant differences in the various groups and under changing conditions; the weight of the left adrenal in the guinea pig is greater than that of the right gland, which is encroached upon by the liver. Therefore, care had to be taken to compare only glands from the same side.

The individual and average weight values are recorded in Fig. 5. The average weight in the untreated tuberculous guinea pig was found to be greater than that in the normal animal. This elevation was not influenced by streptomycin therapy, either during the treatment or in the survival period. Cortisone inhibited the weight increase in tuberculous animals without depressing it below the normal level. When cortisone was discontinued, the adrenal weight corresponded to that in normal animals; but after a survival period of thirty-six days, the average weight of the gland was found to be at the same level as in the untreated tuberculous animals. The same phenomenon occurred in the group which received cortisone together with streptomycin. The evidence thus gained indicates that a weight increase of the adrenal gland, which occurs regularly in tuberculous guinea pigs, is suppressed by cortisone therapy, but only as long as the injections are continued.

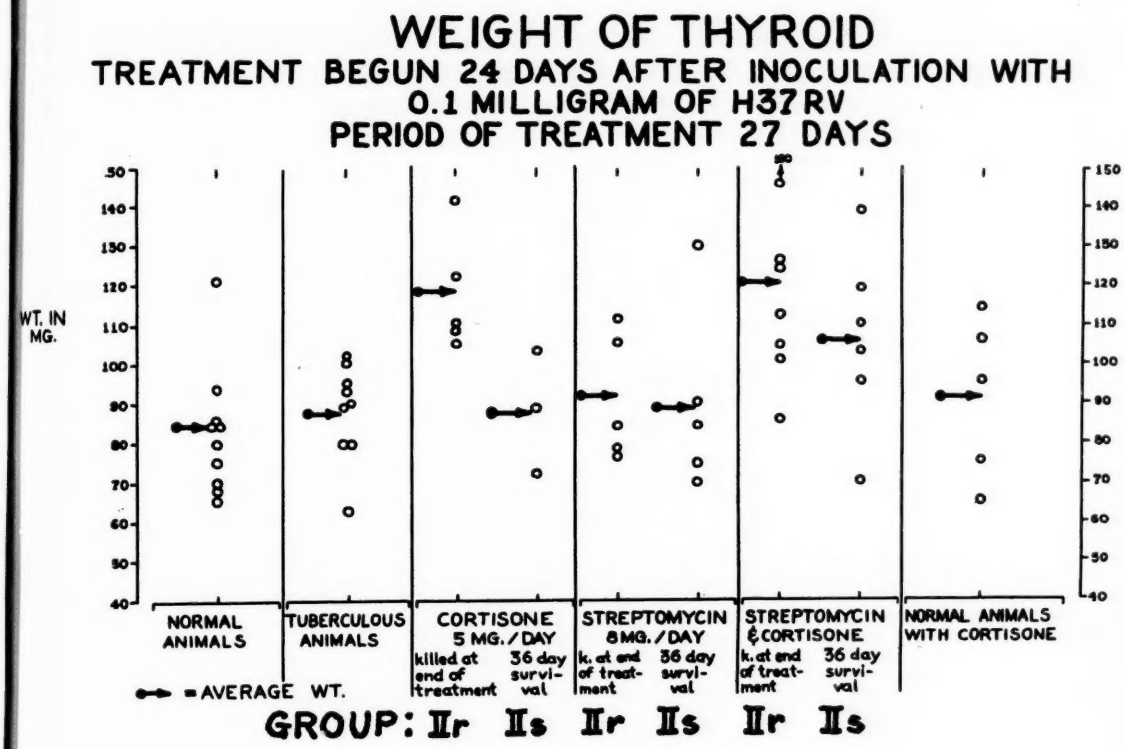
WEIGHT OF THYROID
TREATMENT BEGUN 24 DAYS AFTER INOCULATION WITH 0.1 MILLIGRAM OF H37RV
PERIOD OF TREATMENT 27 DAYS
WT. IN MG.
NORMAL ANIMALS
TUBERCULOUS ANIMALS
CORTISONE 5 MG./DAY
killed at end of treatment
36 day survival
STREPTOMYCIN 8 MG./DAY
k. at end of treatment
36 day survival
STREPTOMYCIN & CORTISONE
k. at end of treatment
36 day survival
NORMAL ANIMALS WITH CORTISONE
= AVERAGE WT.
GROUP: IIr IIs IIr IIs IIr IIs

Fig. 6.

On the cut surface of the glands distinct anatomic differences were noticeable. The white, fat-containing, peripheral part of the cortex is widened in tuberculous guinea pigs (Fig. 7, *C*) and usually not as sharply set off against the central parts of the organ as in normal animals (Fig. 7, *A*). Streptomycin therapy did not alter this change (Fig. 7, *D*). In uninfected cortisone-treated animals the white line was narrower and somewhat sharper than in the normal

pigs (Fig. 7, *B*). In tuberculous animals cortisone alone or in combination with streptomycin approximately kept the appearance of the cut surface similar to that in the normal group (Figs. 7, *E* and *F*). Comparison of the average weight of the adrenal gland with the gross anatomic appearance of the cortex in each group of animals shows parallelism in their changes, both under the influence of tuberculous infection, of cortisone, and of their combination. The size of the adrenal per weight of the animal in the guinea pig is known to be the greatest of all mammals. The increase of its size in tuberculous guinea pigs was first suggested by Sergent[8] and later by Webb[9] and Crowe and Wislocki.[10] The latter

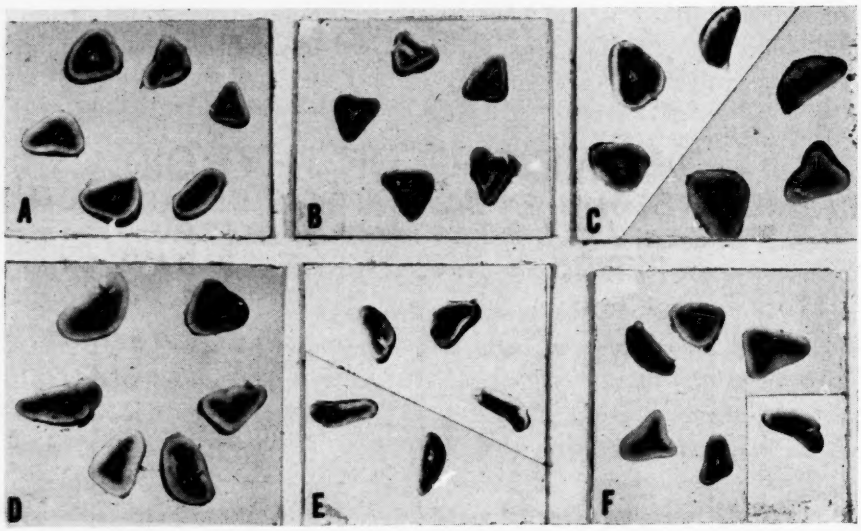

Fig. 7.—Cut surface of adrenal glands. *A*, Healthy guinea pigs. *B*, Healthy guinea pigs treated with cortisone and killed at termination of treatment. The white part of the cortex is narrowed, its inner border sharply delineated. *C*, Tuberculous guinea pigs, untreated. The white part of the cortex is widened, its inner border of more diffuse or ragged outline. *D*, Tuberculous guinea pigs treated with streptomycin. The glands have the same average appearance as in the untreated animals in *C*. *E*, Tuberculous guinea pigs treated with cortisone and killed at termination of treatment. Appearance similar to that seen in healthy untreated animals. (The apparent reduction in the size of the glands as compared with the healthy animals is due to the fact that all but one of them are the right adrenal which in the guinea pig is uniformly smaller and flatter than the left.) *F*, Tuberculous guinea pigs treated with both streptomycin and cortisone; animals killed at termination of treatment. Appearance similar to that seen in healthy untreated animals.

authors theorized on the probable role of the adrenal cortex and made an early suggestion for the use of adrenocortical extract. The histologic findings in the adrenal glands substantiated the impressions gained from gross anatomic inspection. The sections stained for fat showed the following changes in the various experimental groups when compared with glands of normal animals. In the latter the fat-enriched part of the cortical area mostly comprises the peripheral part of the zona fasciculata (Fig. 8, *A*). Only a few lipoid droplets are found in the zona glomerulosa.[11] In a majority of cortisone-treated normal animals this peripheral area appeared narrowed and more sharply demarcated toward the central part. The zona fasciculata appeared more densely loaded with fat, but as a rule a smaller part of it was involved than in untreated animals (Fig.

8, *B*). In the tuberculous animals the changes tended to occur in the opposite direction. In most of the glands the fat-containing cells extended deep into the zona fasciculata, often occupying it entirely and extending into the zona reticularis (Fig. 8, *C*). The pattern of the lipoid-containing cells appeared more loosely knitted; in some specimens a denser inner layer of fat-containing cells had accumulated at some distance from the peripheral part of the zona fasciculata (Fig. 8, *D*). In the glands of cortisone-treated tuberculous animals killed at the

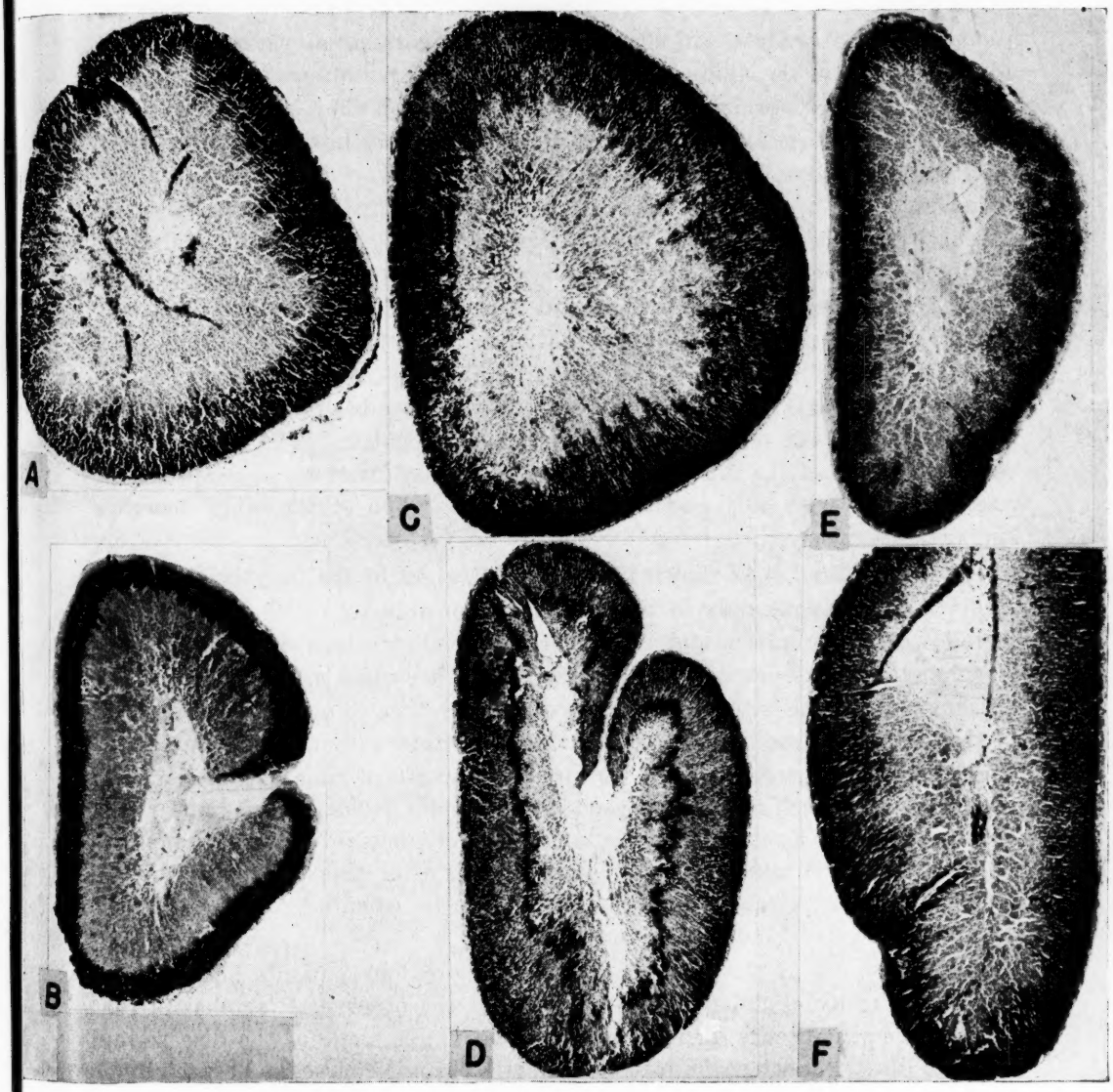

Fig. 8.—Histologic section of adrenal gland stained for fat (scarlet red stain). *A*, Normal. *B*, Normal, treated with cortisone. *C*, Tuberculous, untreated. *D*, Tuberculous, untreated. *E*, Tuberculous, treated with cortisone, killed at termination of treatment. *F*, Tuberculous, treated with cortisone, thirty-six day survival after treatment.

termination of treatment, the average histologic picture did not differ from that in normal animals (Fig. 8, *E*). In the groups which were allowed to survive for thirty-six days the findings for the greater part resembled those in untreated tuberculous animals (Fig. 8, *F*). The sex of the animals did not seem to play a role in these developments. Like the observations on changes of weight and on gross pathologic examination, the histologic findings lead to the conclusion that cortisone in tuberculous guinea pigs inhibits the changes in the adrenal gland produced by tuberculous infection.

Cain and Harrison,[12] in their recent investigations on variations in the adrenal cortex in the albino rat, found that the distribution of lipoids in the adrenal cortex of normal rats differs widely. When they killed rats of the same litter on successive days, they observed variations in the adrenal pattern similar to those seen by us in the guinea pigs of the various groups of our experiment. When rats of the same litter were killed simultaneously, the adrenals rsembled each other. These investigators suggested that the changes may normally occur in a cycle of regular or irregular periodicity. If this be so, the changes observed in our experiment would have to be interpreted as dynamic rather than static ones. It would seem then, that tuberculous infection accelerates a cycle in the shift of lipoids from the outer zona fasciculata toward the zona reticularis, while cortisone delays it and tends to arrest the fat-containing cells in the outer layers of the zona fasciculata. The fact that the changes in each of the different groups were not uniform, but overlapping between groups, with only emphasis on one observation or the other, tends to support this theory.

Thyroid Gland.—As shown in Fig. 6 the weight of the thyroid gland was significantly changed only in the two groups of animals which were both infected with tuberculosis and given cortisone, either alone or together with streptomycin. The glands in both these groups increased markedly in weight, a change which was reversible as shown by the return to normal of the glands in the animals allowed to survive for thirty-six days without treatment. Normal animals receiving cortisone for the same length of time and in the same dose showed no such response of the thyroid gland. It would thus appear that both the stress of a relatively long-standing tuberculous infection in the guinea pig and cortisone administration were necessary for this particular thyroid response. Histologically there appeared to be no essential difference in the appearance of the glands of the various groups.

Enlargement of the thyroid gland in tuberculous patients has been described by many clinicians. The majority of the observers[13] emphasize that the enlargement occurs during the early and active stage of the disease and tends to disappear when the infection takes a chronic course, and especially as the patient's condition improves. Webb[9] described enlargement of the gland in tuberculous guinea pigs. Animals from which he had removed either all or much of the thyroid tissue died earlier and had more extensive tuberculosis than the control animals. He concluded that "this increase in the size and func-

tion of the thyroid is a phase in the marshalling of the body's defensive forces against the invading disease." The mechanism of this thyroid response leaves room for speculation but will remain unexplained until enough facts are known about the interrelationship between the thyroid and adrenal glands.

Pituitary Gland.—The pituitary glands which were removed from the guinea pigs in some of the treated groups show histologic deviations from the gland in normal animals. They are demonstrable through recently developed special stains[14] which permit differentiation between two classes of basophile cells. The result of this study, which will require detailed cell counts and demonstration by colored photomicrographs, will be reported separately.

The gonads of male animals were not obtained in sufficient numbers to be included in this investigation.

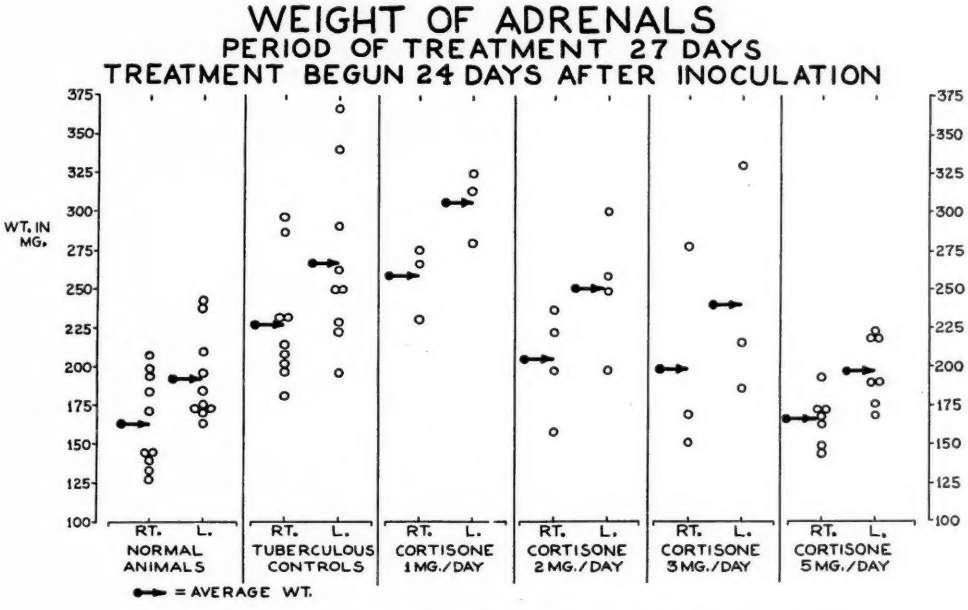

Fig. 9.—The effect of various doses of cortisone on the tuberculous guinea pig.

Effects of Various Doses of Cortisone.—The results of the experiment which was carried out to determine the daily cortisone dose which could promise significant effect in the guinea pig showed insufficient variations as judged by the main clinical criteria. The daily dose of 1 mg., 2 mg., and 3 mg. produced only slight and questionable depressions of the tuberculin reaction, the blood sedimentation rate, and the eosinophile count. Fig. 9 shows the cortisone effect on the size of the adrenal gland by the various dosages. In the group treated with 1 mg. daily there was a slight but insignificant increase of the average adrenal weight over the increase in untreated tuberculous animals. Neither the 2 mg. nor the 3 mg. dose reduced the weight increase to the average level found in normal guinea pigs. Five milligrams daily was the minimal dose which reliably exerted this average inhibitory effect.

SUMMARY AND CONCLUSIONS

Prolonged administration of cortisone (5 mg. daily for twenty-four days) to tuberculous guinea pigs promoted the development of tuberculosis, especially during the early stages of the disease. Cortisone interfered with the therapeutic effect of streptomycin. Cortisone inhibited specific hypersensitivity to about 50 per cent during the period of injections. The hypersensitivity restored itself after the injections were discontinued. The blood sedimentation rate in untreated guinea pigs spontaneously returned to preinfection levels after an initial rise which roughly concurred with the beginning of hypersensitivity. This initial elevation was inhibited by cortisone as well as by streptomycin therapy. Eosinophile cells in the blood disappeared entirely in the majority of cortisone-treated guinea pigs. The enlargement of the adrenal gland which occurs under the stress of tuberculous infection in guinea pigs was inhibited by cortisone during the treatment period but recurred in animals which were permitted to survive the injections. On macroscopic examination the light part of the adrenal cortex appeared narrowed in animals killed immediately after cessation of treatment. Histologically these changes expressed themselves as a loosening and widening of the fat-containing zona fasciculata in tuberculosis, and as a tightening and narrowing under the influence of cortisone. These findings may be part of a normal cycle of changes, altered in its course by cortisone. The thyroid gland was significantly increased in weight in cortisone-treated tuberculous guinea pigs killed at the termination of treatment. It was normal in animals which were permitted to survive.

The daily dose of 5 mg. of cortisone was found to be the minimum effective dose for guinea pigs in a separate experiment in which various doses were employed.

We are indebted to Merck & Co., Inc., Rahway, N. J., for their generosity in supplying a large part of the cortisone used in these experiments.

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LABORATORY METHODS

PHOTOMETRIC DETERMINATION OF AMYLASE ACTIVITY IN PANCREATIC JUICE

JEROME M. WALDRON, M.D.
PHILADELPHIA, PA.

THE present report is a description of a simple method for the determination of pancreatic amylase activity. In 1943 Riggs and Stadie[1] reported a photoelectric method for the determination of peptic activity in gastric juice. The principle of this test is the measurement of the decrease of turbidity in time of a standard egg white suspension. Peptic activity is expressed as the velocity constant of the monomolecular digestion of the protein substrate. A modification of this procedure by Friedman[2] for trypsin in pancreatic juice has placed the determination of the activity of two digestive enzymes on one principle.

Since the digestion of a starch suspension by pancreatic amylase is a monomolecular process[3, 4] the use of this technique for determining its activity awaited only the production of a stable starch suspension. Such a suspension of starch is now readily available, inexpensive, and completely stable.

MATERIALS

Substrate.—A standard suspension of starch is commercially available,* consisting of 10 per cent starch suspension in water with 4 to 4.2 per cent sodium chloride and 0.5 per cent pine oil added. The pine oil prevents bacterial and fungicidal decomposition of the starch. Such a standard suspension is stable at room temperature indefinitely. The suspension will withstand freezing and thawing, and remain stable.

Sodium Chloride.—A 1.0 molar sodium chloride solution is used to insure complete activity of the amylase. The extent of amylolysis is related to the amount of sodium chloride present below a certain concentration of sodium chloride in the digestion mixture.[5]

Buffer Solution.—This is prepared by dissolving 7.62 Gm. of anhydrous potassium dihydrogen phosphate and 20.45 Gm. of disodium hydrogen phosphate in distilled water to a total volume of 1 liter. The resulting solution is a buffer of pH 7.2 which is optimum for pancreatic amylase[6] and duodenal contents.[7]

Standard Turbidity Curve.—The relation of turbidity to starch concentration in the digestion mixture is shown in Fig. 1. It is obtained by making the complete substrate mixture from the standard substrate as in the described procedure with the exception that 0.1 normal hydrochloric acid is used instead of buffer solution in order to prevent activity of the amylase. To this is added 1 ml. of diluted pancreatic juice. With this as 100 per cent substrate, serial dilutions are made and the percentage substrate is plotted against turbidity using a Filter 42 (blue violet) for the Klett-Summerson instrument. The plot of this curve will be constant as long as the same instrument is used.

From the Department of Physiology, Jefferson Medical College.
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*Obtainable from Zippy Products Company, Lansdowne, Pa.

PROCEDURE

To 0.5 ml of the standard starch suspension is added 1 ml. of 1.0 molar sodium chloride and 3.5 ml. of phosphate buffer. This complete substrate mixture is then placed in a water bath at 38° C. ±0.1° until warm. Then the mixture is shaken, and 1 ml. of pancreatic juice diluted 1:100 in phosphate buffer is added rapidly and immediately timed with a stop watch. The mixture is again shaken and put in the water bath and at exactly one minute the turbidity is read in a Klett-Summerson photoelectric instrument using Filter 42. The mixture is replaced in the water bath. At exactly three minutes the turbidity is again determined.

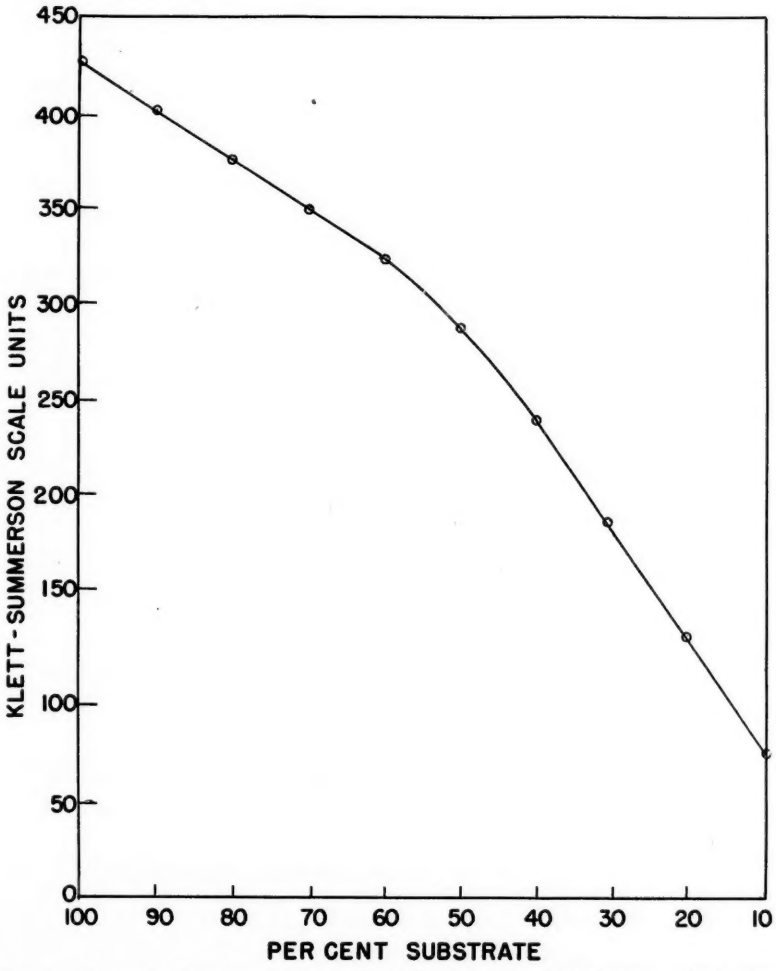

Fig. 1.—Concentration curve for a Klett-Summerson photoelectric colorimeter scale with a No. 42 filter. One hundred per cent represents the concentration of substrate plus 1 ml. of diluted pancreatic juice.

From these two turbidity readings the concentration of substrate at one minute and three minutes is determined from the standard turbidity curve. Then the velocity constant (k) is calculated from the formula (1):

$$k = (1/t_2 - t_1) \times 2.3 \log c_1/c_2 \text{ or } k = 1.15 \log c_1/c_2$$

In comparing this procedure with a saccharinogenic method[8] on duodenal contents of 100 normal patients in the fasting state the velocity constant ranged from 0.107 to 0.164 with 75

per cent of the samples above 0.130. This spread of normal values for the velocity constant is consistent with the spread of the saccharinogenic method which ranged from 29 to 100 amylase units with 75 per cent of the samples above 33 amylase units. The normal value will, of course, vary if pancreatic juice is obtained as a result of stimulating the pancreas and the direction of change will depend on whether the stimulus promotes primarily water or enzyme secretion. Therefore, normal values must be established for each of the many ways of stimulating the pancreas.

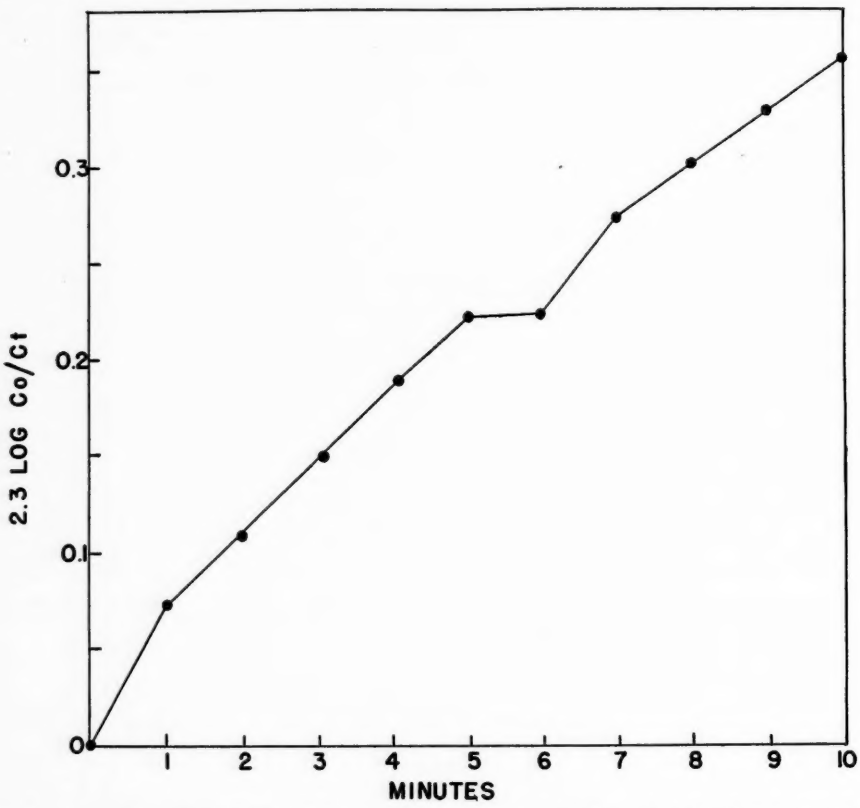

Fig. 2.—The rate of hydrolysis of the standard starch suspension when incubated with 1 ml. of a mixture of alpha amylase and beta amylase. The concentration of each enzyme is 1:1,000.

DISCUSSION

The validity and reproducibility of this procedure depends upon the stability of the standard substrate, the presence of sufficient substrate, the proper conditions for the reaction, the correlation of turbidity and concentration of substrate, and the monomolecular nature of the digestion of starch by pancreatic amylase in this method. All of these conditions have been fulfilled in the present procedure.

As stated previously, the salt concentration and pH of the mixture is optimal. The standard starch suspension is not only stable but provides an excess of starch for digestion. The 50 mg. of starch present has proved adequate in all determinations to date. In addition this concentration of substrate produces a turbidity in the optimal range of the Klett-Summerson instrument.

In over 200 determinations the readings on the Klett-Summerson scale of the complete substrate has been consistently 450 scale units (within the accuracy of the instrument), and when 1 ml. of diluted pancreatic juice is added it is 425. This is taken as representing 100 per cent substrate. When pure pancreatic juice is used the initial turbidity is always 425. Clinically, pancreatic juice is obtained by collecting duodenal contents. In this case a blank should be prepared by diluting 1 ml. of the 1:100 duodenal contents with 5 ml. of phosphate buffer. The reading for the blank is then subtracted from the turbidity readings at one minute and three minutes before calculating the velocity constant.

A mixture of alpha and beta amylase (Nutritional Biochemicals Corporation) to give a concentration of 1:1,000 of each enzyme was used to determine whether this reaction is monomolecular. In Fig. 2 time is plotted against log c_o/c_t. It is evident that from the end of the first minute to five minutes the reaction behaves as a monomolecular one. In the described procedure turbidity is read at one minute and three minutes. In determinations of amylase activity of pure pancreatic juice ranging from poor activity to very high activity these time readings have proved satisfactory. The reason for the nonlinearity in the first minute is not known. It is probably related to inadequate immediate mixing, or perhaps the substrate is not completely homogeneous. The reason for the appearance of the curve after five minutes is unexplained at present, but it is a consistent finding and occurs with pancreatic juice also.

For any individual sample of pancreatic juice the determinations are reproducible. The range of error in reading turbidity on the Klett-Summerson instrument is ±5 scale units. This represents 2 per cent error in substrate concentration. This figure refers to highly active pancreatic juice; when the sample is of low activity the turbidity readings are completely reproducible. Table I gives sample readings of the same pancreatic juice on four different substrate preparations.

TABLE I. DEGREE OF REPRODUCIBILITY*

TIME (MIN.)	SUBSTRATE I	SUBSTRATE II	SUBSTRATE III	SUBSTRATE IV
1	325	325	325	325
2	298	298	296	294
3	284	280	284	280
4	250	250	250	250

*Four different preparations of standard substrate are digested by the same sample of pancreatic juice. The readings of different aliquots during digestion do not vary more than five scale units at any one time.

This turbidimetric method for the determination of the activity of pancreatic amylase is a simple, rapid, inexpensive, and reproducible method. It has the advantage that a comparison can be made with other enzymes because the activity of pepsin, trypsin, and amylase can be measured by the physicochemically significant velocity constant of the reaction.

SUMMARY

A simple, rapid, and reproducible procedure for the determination of the amylolytic activity of pancreatic juice is described.

Enzyme activity is measured as the velocity constant of the monomolecular hydrolysis of a standard, stable starch suspension under specified optimal conditions.

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GAMMA GLOBULIN DETERMINATIONS

Comparative Values Obtained by Turbidimetric and Electrophoretic Methods

William E. Ricketts, M.D., Kenneth Sterling, M.D., and
Robert S. Levine, B.S.
Chicago, Ill.

TURBIDIMETRIC methods of estimating the gamma globulin concentration in the sera have been devised recently.[1, 2] An evaluation of these procedures in comparison with electrophoretic values is undertaken in the present study.

METHODS

Electrophoretic analysis of the serum proteins was done with the standard apparatus and technique described by Longsworth[3] as reported in a previous study.[4] The turbidimetric methods used for measuring gamma globulin were the zinc sulfate test of Kunkel[1] and the ammonium sulfate-sodium chloride test of de la Huerga and Popper.[2] The results are expressed in grams of gamma globulin per cent following the conversion formula described by these authors.

MATERIAL

Simultaneous determinations of gamma globulins by the turbidimetric and the electrophoretic methods were done in fifty different cases distributed as follows: normals, seven; portal cirrhosis, twenty; biliary cirrhosis, eleven; acute virus hepatitis, three; and recovered virus hepatitis, seven.

RESULTS

The values obtained are given in Table I. While there was an approximation between the turbidimetric and electrophoretic values in normal individuals and in recovered virus hepatitis, the results were not parallel in cases of portal and biliary cirrhosis, with differences higher than 100 per cent in several cases. This is illustrated in Fig. 1. In most cases (thirty-nine of fifty) the electrophoretic values were higher than the values obtained by the turbidimetric methods.

DISCUSSION

The simplicity of the turbidimetric methods recently devised to measure serum gamma globulin makes possible their consideration as routine clinical procedures. Previous studies by de la Huerga and co-workers[5] have compared the findings of the two turbidimetric methods with the electrophoretic determinations of gamma globulin. Statistically the zinc sulfate method correlated less well with the electrophoretic partition of gamma globulin and the expected error was almost twice that obtained using the ammonium sul-

From the Frank Billings Medical Clinic, the Department of Medicine, The University of Chicago.
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TABLE I. COMPARATIVE VALUES OF GAMMA GLOBULIN BY TURBIDIMETRIC AND ELECTROPHORETIC METHODS, EXPRESSED IN GRAMS PER CENT OF GAMMA GLOBULIN

SUBJECTS		ELECTROPHORETIC METHOD	ZINC SULFATE METHOD		AMMONIUM SULFATE-SODIUM CHLORIDE METHOD	
		VALUES	VALUES	DIFFERENCE $ZnSO_4$ ELECTRO	VALUES	DIFFERENCE AMM.-ELECTRO
Normal	1	0.96	0.97	+0.01	0.67	−0.29
	2	0.68	0.77	+0.09	0.66	−0.02
	3	0.77	0.82	+0.05	0.82	+0.05
	4	0.99	0.99	0	0.86	−0.13
	5	0.91	0.77	−0.14	0.72	−0.19
	6	1.11	1.01	−0.10	0.86	−0.25
	7	1.06	0.98	−0.08	0.72	−0.34
Mean		0.93	0.90	−0.03	0.76	−0.17
Portal cirrhosis	1	1.81	0.76	−1.05	1.48	−0.33
	2	1.40	0.99	−0.41	1.12	−0.28
	3	1.17	0.84	−0.33	0.99	−0.18
	4	0.88	0.69	−0.19	0.76	−0.12
	5	2.61	1.75	−0.86	1.41	−1.20
	6	1.01	0.88	−0.13	1.18	+0.17
	7	1.00	0.86	−0.14	0.80	−0.20
	8	0.90	0.95	+0.05	0.99	+0.09
	9	0.70	0.66	−0.04	0.83	+0.13
	10	2.06	1.09	−0.97	1.47	−0.59
	11	2.42	1.16	−1.26	1.50	−0.92
	12	1.84	0.98	−0.86	1.13	−0.71
	13	2.10	1.05	−1.05	1.39	−0.71
	14	1.60	0.89	−0.71	1.28	−0.32
	15	2.59	1.27	−1.32	1.47	−1.12
	16	1.40	1.34	−0.06	1.35	−0.05
	17	2.34	1.11	−1.23	1.51	−0.83
	18	2.37	1.57	−0.80	1.68	−0.69
	19	1.49	0.86	−0.63	1.12	−0.37
	20	1.21	0.86	−0.35	0.86	−0.35
	21	1.78	1.07	−0.71	1.63	−0.15
	22	2.13	1.52	−0.61	1.69	−0.44
Mean		1.67	1.05	−0.62	1.26	−0.41
Biliary cirrhosis	1	1.63	0.93	−0.70	1.53	−0.10
	2	1.12	0.70	−0.42	0.99	−0.13
	3	1.33	0.55	−0.78	0.67	−0.66
	4	1.23	0.89	−0.34	0.96	−0.27
	5	0.95	0.61	−0.34	0.75	−0.20
	6	2.74	1.36	−1.38	1.97	−0.77
	7	1.63	0.92	−0.71	1.53	−0.10
	8	0.99	0.80	−0.19	1.36	+0.37
	9	1.12	0.70	−0.42	0.99	−0.13
	10	1.14	0.85	−0.29	0.93	−0.21
	11	1.08	0.83	−0.25	0.80	−0.28
Mean		1.36	0.83	−0.53	1.13	−0.23
Virus hepatitis Acute	1	1.40	0.89	−0.51	0.89	−0.53
	2	0.97	0.62	−0.35	0.88	−0.09
	3	0.89	0.99	+0.10	0.83	−0.06
Recovered	4	1.05	0.89	−0.16	0.83	−0.22
	5	1.07	1.03	−0.04	0.90	−0.17
	6	0.96	0.82	−0.14	0.81	−0.15
	7	1.04	0.89	−0.15	1.02	−0.02
	8	1.43	1.04	−0.39	1.12	−0.31
	9	1.18	0.84	−0.34	1.01	−0.17
	10	1.49	0.80	−0.69	0.83	−0.66
Mean		1.15	0.88	−0.27	0.91	−0.24

fate-sodium chloride reagent. This was attributed[6] to several factors other than the gamma globulin concentration of the serum albumin and lipid. The albumin was said not to influence the ammonium sulfate-sodium chloride turbidity and correction was made for the lipid factor. In the present study both of the turbidimetric methods showed appreciable deviations from electrophoretically determined gamma globulin values. The discrepancies were often considerable (50 to 100 per cent or greater) in sera from patients with portal and biliary cirrhosis, while there was usually agreement within 20 per cent or less in normal sera. The magnitude of these discrepancies illustrated in Fig. 1 and Table I make it seem unwarranted to express to two decimals the values for gamma globulin by either of the turbidimetric methods. Pending further comparisons of these techniques with electrophoresis in other laboratories, it is felt they should not be regarded as exact equivalents of the electrophoretic gamma globulin. It is not unlikely that in the near future more accurate, simplified procedures will be available.

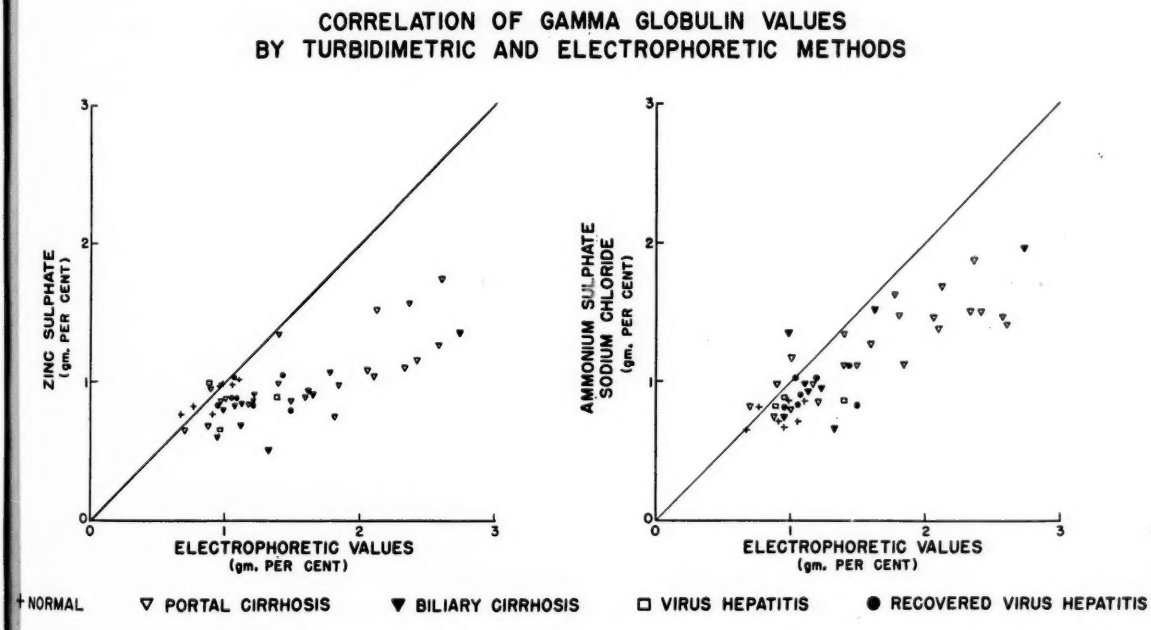

Fig. 1.

SUMMARY

Simultaneous determinations of gamma globulin values by electrophoresis and by the zinc sulfate and the ammonium sulfate-chloride turbidity methods were done in fifty individuals. In normal subjects these determinations usually corresponded within 20 per cent. However, in portal cirrhosis and biliary cirrhosis there were marked differences between turbidimetric and electrophoretic values.

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THE MICROESTIMATION OF ANTIBODIES WITH ONE MILLILITER SAMPLES OF ANTISERA OF LOW ANTIBODY CONTENT

ROBERT C. KRUEGER, PH.D., AND MICHAEL HEIDELBERGER, PH.D.
NEW YORK, N. Y.

AMONG the procedures now available for the quantitative estimation of antibodies in immune sera, the micro-Kjeldahl method for the determination of antibody nitrogen[1] and the modification of Markham[2] are most easily applicable to the range of values commonly found in hyperimmune sera of animals. In human subjects after vaccination or recovery from infectious disease the amounts of antibody present in the serum are usually much smaller, so that a more sensitive method is required. This was devised by allowing the mixtures of antigen and antiserum to stand for longer periods than had been customary, with precautions to ensure sterility, and by the use of a colorimetric method for the estimation of nitrogen such as a modification[3] of that employing the Folin-Ciocalteu reagent.[4] In this way, after removal of the nonspecific proteins by washing, residues of specific precipitate containing as little as 1 μg of nitrogen could be estimated by the color produced with the reagent. The method, however, is based upon the use of a blank and duplicate samples of serum as large as 4 milliliters. Because of the size of the tubes and the consequent volume of the washings it is probable that values of antibody nitrogen of the order of 10 μg or less are too low, owing to an appreciable solubility of the specific precipitate.[5] It therefore seemed desirable to attempt further refinements of technique which would permit the use of smaller tubes and 1 ml. portions of serum. This has been accomplished, with results that have usually agreed closely with those on 4 ml. samples run for comparison at the same time.

The modifications of method herein described make available for the first time a method yielding antibody content in weight units in sera of children or in weak antisera of animals in instances in which only small volumes of sera can be spared.

EXPERIMENTAL

One milliliter portions of serum or serum dilution are pipetted into 2 or 3 ml. conical Pyrex centrifuge tubes marked at any convenient volume slightly above 1 ml., such as 1.1 milliliters. Based on a preliminary test, a slight excess of antigen is added and the mixtures are stirred with a fine glass rod. Precautions to ensure sterility are used throughout, the tubes are capped with sterile rubber caps, and sera and solutions are kept cold. The tubes are stirred daily by placing them in a thick rubber tube mounted on the

From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital in the City of New York.

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shaft of an inverted small stirring motor and operating the motor intermittently and cautiously. A loop of wire attached to the ring stand holding the motor serves as a support for the glass tube. Preliminary experiments showed that under these conditions four days in the refrigerator are usually sufficient for quantitative precipitation of the antibody, although slightly higher results for anti-C, the antibody to the somatic polysaccharide of pneumococcus, were obtained after eight days. In the present studies a period of eight days was allowed. The tubes are centrifuged in the cold and the supernatants poured off. The small amount of supernatant remaining in the narrow conical tip of each tube is made to run down the wall of the tube inverted at an angle by touching it with a fine glass rod of 1 to 1.5 mm. diameter. The supernatants are treated with more of the same antigen in order to make certain that all of the antibodies are precipitated, and the analysis is repeated if necessary. The precipitates are broken up and smoothed out as well as possible with a glass rod, washed three times with 1 ml. of chilled saline, and centrifuged. The last drop of wash fluid over the precipitate in the tip of each tube is removed each time as described.

Normal NaOH, 0.165 ml., is added to dissolve the precipitate and the contents of the tube are brought to the 1.1 ml. mark with water. Thorough mixing is accomplished on the stirrer described. The solutions are allowed to stand one hour after which 1.0 ml. aliquots are pipetted into small test tubes. Five-tenths milliliter of 0.1 per cent $CuSO_4$ solution and 3.0 ml. of 12.5 per cent Na_2CO_3 solution are added and the mixtures allowed to stand twenty minutes. Five-tenths milliliter of Folin reagent is added dropwise and the blue color is read after twenty minutes in 1.00 cm. matched cuvettes in a Beckman spectrophotometer at 650 millimicrons.

In preliminary experiments with various protein solutions, it was shown that the extinction coefficient (optical density ÷ [cell-length × protein concentration]) determined at 650 mμ in the Beckman spectrophotometer was identical with the extinction coefficient determined at 650 mμ in the Coleman instrument used in the earlier method,[3] so that a factor could be calculated readily with which to multiply the color value then found. The volume in the earlier method was 10 ml. while that used in the present modification is 5 milliliters. The cell length in the Coleman instrument is 1.30 cm. and in the Beckman, 1.00 cm., therefore,

$$\text{Beckman factor} = \text{Coleman factor} \times \frac{1.3}{2}$$

An example is given in Table I.

Results of the analysis of several sera are shown in Table II.

Antibody N or total N might also be directly measured in the ultraviolet range on solutions of specific precipitates,[7] or by the ninhydrin reaction.[8]

DISCUSSION

Although it is generally possible to obtain 13 to 15 ml. of human sera for analysis of the antibody content, as required in the micromethod hitherto used,[3] this is usually difficult in the case of small children and impossible with small laboratory animals or when it is desired to make frequent test bleedings of rabbits, for example. The present refinement of the analytic method to a point at which only about 3.5 ml. of serum are required for blank and duplicate precipitin analyses should therefore be of considerable utility. In general the modified micromethod with 1 ml. samples and small tubes gives values agreeing reasonably well with those obtained by the method devised for 4 ml. samples of serum. Slightly lower values are more frequent than higher ones, and this is probably due to the slight solubility of specific precipitates,[5] an effect which looms larger with smaller amounts of precipitate nitrogen.

TABLE I. ANTIBODY NITROGEN IN HUMAN SERUM 91_1 (3D)

VOLUME FOR ANALYSIS:	4 ML.	1 ML.
	First addition of S I	
Vol. of soln. of sp. ppt.:	2.5 ml.	1.1 ml.
Aliquot for analysis:	2.0 ml.	1.0 ml.
	Optical density 0.607*	Optical density 0.255*
	$0.607 \times 64.2\dagger \times \frac{2.5}{2.0} = 48.7\ \mu g$	$0.255 \times 41.7\ddagger \times \frac{1.1}{1.0} = 11.7\ \mu g$
	Second addition of S I	
	Optical density 0.124*	Optical density 0.053*
	$0.124 \times 64.2 \times \frac{2.5}{2.0} = 10.0\ \mu g$	$0.053 \times 41.7 \times \frac{1.1}{1.0} = 2.4\ \mu g$
Total N found	59 μg	14.1 μg ($\times 4 = 56\ \mu g$)

S I, Specific polysaccharide of Type 1 pneumococcus.

*After subtraction of color value of precipitate in serum control without antigen.

†Factor for antibody N obtained by comparison of color densities and micro-Kjeldahl values.

‡$64.2 \times \frac{1.3}{2}$

TABLE II. COMPARISON OF VALUES FOR ANTIBODY N FOUND WITH 4 ML. AND WITH 1 ML. SAMPLES OF SERA

			ANTIBODY N IN SERUM			
SERUM	NUMBER	ANTIGEN	4 ML. (μG)	1 ML. (μG)	1 ML. × 4 (μG)	FROM SERIES E (3D) 4 ML. (μG)
Human	89_1	C III	34	7.2, 6.7	29, 25	30
Human	89_1	S I	30	6.2, 7.2*	25, 29	33
Human	90_1	C III	30	7.5	30	26
Human	90_1	S I	32	7.0	28	33
Human	91_1	C III	43	10.3	41	42
Human	91_1	S I	59	14.1	56	46, 65
Human	94_1	C III	82†	22.8	89	89
Human	94_1	S I	65	13.5	54‡	70
Human	87_1	C III	60	9.5	38‡	80
Human	87_1	S II	50	13.2	53	45
Human	90_3	C VI	32	7.7	31	28‖
Human	90_2	S III	29	5.9	24	22‖
Rabbit	FK5	Egg albumin	45§	11.5§	46§	

C III, Somatic C polysaccharide of pneumccoccus, isolated as by-product from a Type 3 culture.

*One determination lost.

†Probably low because color was read at too high a point on optical density curve, where relation was no longer linear.

‡Reason for low result unknown.

§Total N precipitated.

‖From reference 6.

SUMMARY

Refinement of an earlier microcolorimetric method for the quantitative estimation of antibody in human sera and low-grade antisera in animals now makes it possible to use as little as 3 ml. of serum for a blank and duplicate determinations.

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THE EFFECT OF SERUM PROTEINS ON THE DETERMINATION OF CHLORIDE BY MERCURIMETRIC TITRATION

JOSEPH S. ANNINO*
NEW HAVEN, CONN.

INTRODUCTION

THE determination of chloride in serum, without the preliminary removal of proteins, by direct titration with mercuric nitrate solution as described by Schales and Schales,[1] holds the advantage of simplicity over the widely used iodometric[2] or modified Volhard[3] techniques which employ protein removal before titration. Because of the known propensity of protein to bind mercury and other heavy metals, it was thought desirable to investigate the effect of proteins on chloride recovery by mercurimetric titration. To this end, comparative analyses were performed by the mercurimetric and modified Volhard methods on standard chloride solutions in the presence of added serum proteins. The results indicate that serum proteins produce falsely high chloride values with the mercurimetric titration.

EXPERIMENTAL

Fifty sera were analyzed for chloride content by the mercurimetric and the thiocyanate methods. Forty-seven of these sera gave higher results with the mercurimetric titration. The largest discrepancy was 14.8 meq. per liter, the mean deviation was 6.4 meq. per liter, and the standard deviation from this mean was ± 3.9 meq. per liter.

Standard chloride solutions were prepared and determined by both methods. The results are given in Table I.

Recoveries were then done on these solutions after the addition of protein. To 1 ml. of a fivefold dilution of the standards, 0.2 ml., 0.4 ml., and 0.6 ml. respectively of 2.5 Gm. per cent albumin solution† were added. The results of these determinations are found in Table I.

It would appear that in the mercurimetric method there is a significant rise in the apparent chloride value, if determined in the presence of serum albumin, but this discrepancy does not necessarily vary with the concentration of albumin.

TABLE I

TEST SOLUTIONS	MERCURIMETRIC TITRATION	THIOCYANATE TITRATION
A = 144.0 meq./L.	144.0	144.4
B = 72.0 meq./L.	72.8	71.8
C = 108.0 meq./L.	109.2	108.8
A + 0.2 ml. albumin	147.5	144.0
B + 0.2 ml. albumin	74.7	72.0
C + 0.2 ml. albumin	112.1	108.4
A + 0.4 ml. albumin	148.0	143.6
B + 0.4 ml. albumin	75.5	71.4
C + 0.4 ml. albumin	114.1	109.0
A + 0.6 ml. albumin	148.8	145.6
B + 0.6 ml. albumin	75.2	72.5
C + 0.6 ml. albumin	115.9	109.6

From the Department of Internal Medicine, Yale University School of Medicine.
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*Present address: Chemistry Laboratory, Massachusetts Memorial Hospitals, Boston, Mass.
†Chloride-free human serum albumin from the American Red Cross.

The effect of human serum globulin* was then studied by the analysis of aliquots of the above-described standard solutions after 0.2 ml. and 0.4 ml. respectively of 3 Gm. per cent globulin solution had been added. The results presented in Table II indicate that globulin has a greater effect on the mercurimetric titration than does albumin. This effect may be related to concentration.

TABLE II

TEST SOLUTIONS	MERCURIMETRIC TITRATION	THIOCYANATE TITRATION
A* + 0.2 ml. globulin	150.1	142.8
B† + 0.2 ml. globulin	78.3	72.1
C‡ + 0.2 ml. globulin	117.4	110.0
A + 0.4 ml. globulin	153.6	
B + 0.4 ml. globulin	79.4	
C + 0.4 ml. globulin	119.1	

*Solution A = 144.0 meq./L.
†Solution B = 72.0 meq./L.
‡Solution C = 108.0 meq./L.

SUMMARY

Comparative chloride analyses were performed by the thiocyanate and mercurimetric titrations on sera and on standard solutions in the presence of added proteins. The results indicate that erroneously high values are obtained by mercurimetric titration in the presence of protein.

CONCLUSION

Removal of proteins is a necessary step in the determination of serum chloride by titration with mercuric nitrate.

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*Chloride free human serum globulin from E. R. Squibb & Sons.

INHIBITION OF BACTERIAL GROWTH BY ADSORBED ANIONIC DETERGENTS

G. Toennies, Ph.D., and D. L. Gallant, A.B., Philadelphia, Pa., and O. Rahn, Ph.D., and B. Iske, B.S., Pocatello, Idaho

I. OBSERVATIONS WITH TEST TUBES (TOENNIES AND GALLANT)

IN BACTERIMETRIC work using *Streptococcus faecalis,*[1] initial results were marred by great variations of growth response in parallel experiments, especially during the first twenty-four hours of growth. These inconsistencies were traced to the method of cleaning the culture tubes. It could be shown that the soap used left inhibiting residues of great persistence; and also with Nacconol (a synthetic detergent) inhibitory effects could not be reliably eliminated by twenty rinsings with cold distilled water.

Fig. 1 shows the results, in groups of ten culture tubes each, of three methods of cleaning. All tubes contained 10 milliliters of a synthetic medium[1] limited by 12 micrograms of threonine. This medium is especially sensitive to growth-inhibiting factors because threonine in limiting concentrations causes a much slower initial growth rate than the other amino acids essential for *S. faecalis* (unpublished observations of Toennies and Gallant). The tubes were simultaneously incubated at 37° to 38° C., and growth was measured by optical density. The final readings (forty-seven hours) show essentially the same average in the Nacconol and the chromic-sulfuric acid series, although earlier the Nacconol treated tubes are definitely inhibited. In seven of the soap-washed tubes growth was almost absent for the first twenty-four hours, but the inhibition is not permanent, as shown by the vigorous response in the second twenty-four hour period in all but two of the strongly inhibited tubes.

Further work showed that one autoclaving with water is more effective than many rinsings with cold water. Our culture tubes are now precleaned by brush, if necessary, then soaked overnight in warm cleaning fluid, followed by thorough rinsings with tap water, one rinsing with distilled water, and one twenty minute treatment at 120° C. in the sterilizer, the tubes being submerged in distilled water. The effectiveness of this treatment is shown in Table I. It is evident that it effectively eliminates all residual effects of soap or Nacconol.

II. OBSERVATIONS WITH PETRI DISHES (RAHN AND ISKE)

The test tube experiments described above were known to us when we encountered inconsistent observations with plate counts of *Streptococcus lactis.* Investigation of the difficulties indicated that we were dealing with the same source of error, namely, the use of detergents in cleaning glassware. Our Petri dishes had been autoclaved in a solution of "Atomic Suds" and then rinsed with cold tap water.

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From the Lankenau Hospital Research Institute and the Institute for Cancer Research, Philadelphia, Pa., and Idaho State College, Pocatello, Idaho.

TABLE I. GROWTH OF OTHERWISE IDENTICAL CULTURES OF *S. faecalis* IN TUBES CLEANED BY DIFFERENT METHODS.*

Cleaning operation			
Washing with water	+	−	−
Washing with soap	−	+	−
Washing with Nacconol	−	−	+
Soaking with chromic-sulfuric acid	+	+	+
Autoclaving with water	+	+	+
Growth (Opt. density, average and standard deviations)			
17 hours	154 ± 4	154 ± 3	155 ± 3
23 hours	197 ± 4	197 ± 2	196 ± 2

*Incubation in water bath (1) at 37.75 ± 0.05° C. Eight tubes in each group. The steps used in each of the three sequences are indicated by + marks.

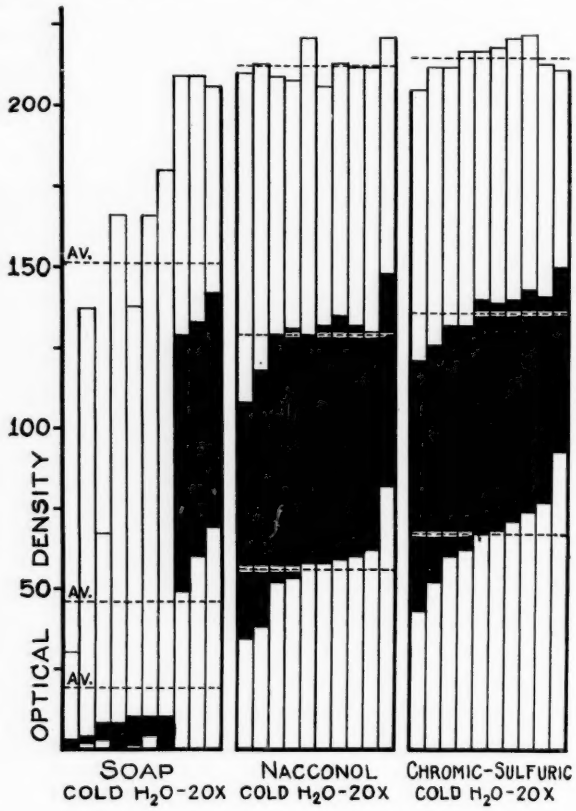

Fig. 1.—Growth of otherwise identical cultures of *S. faecalis* in tubes cleaned by different methods. Each bar represents one tube. The lowest horizontal line on each bar shows growth after nineteen hours; the second line, after twenty-four hours; and the third, after forty-seven hours. The growth increment between nineteen and twenty-four hours is shown in black for visualization purposes. The first group of ten tubes was washed with warm soap suds and rinsed twenty times with cold water, in the second group the initial treatment was with warm Nacconol suds, while the third group was kept in cleaning fluid on the hot steam bath overnight.

While Toennies and Gallant tried to overcome the trouble by oxidation of the detergent and desorption with overheated water, we tried a purely physical approach. The retardation of growth is ultimately due to the reduced surface tension of the detergents which results in a strong adsorption on all surfaces, including those of the glassware. The adsorption may be so strong that repeated

washing will not remove all of it from the surface. This small amount adhering to the surface so tenaciously will gradually spread to any bacteria present because their surface, in relation to volume, is very large indeed.

One way to overcome this surface effect is to provide still larger surfaces, and this was done by placing the detergent-washed Petri dishes into suspensions of bentonite, or of a clay soil, and leaving them there overnight, rinsing them with cold water the next day. Table II gives the plate counts of four methods

TABLE II. COLONY COUNTS OF THE SAME CULTURE OF *Streptococcus lactis* IN PETRI DISHES CLEANED WITH A DETERGENT

		TREATMENT OF PETRI DISHES AFTER CLEANING			
		RINSING WITH TAP WATER		SOAKING WITH	
	DILUTION	ONCE	3 TIMES	BENTONITE	CLAY
Average plate count with average deviation	10^{-6}	98 ± 43	140 ± 36	224 ± 14	211 ± 4
	10^{-7}	7 ± 6	14 ± 2	25 ± 7	23 ± 4

of dishwashing, each number representing the average of triplicate plate counts. The entire set of data was obtained with the same pipette from the same suspension of bacteria; the results are directly comparable.

The differences between the right and left side of the table are sufficient proof that quantitative bacteriological experiments cannot be depended upon when made in glassware cleaned with surface-depressant agents unless measures are taken to counteract any adsorption on the glassware.

SUMMARY

Glassware washed with soap or surface-active detergents is not fit for quantitative bacteriologic experiments because these cleaning agents become adsorbed on the glass surface and adhere there very persistently. Unless they are removed, either by oxidation and desorption autoclaving (I) or by readsorption on other surfaces (II), or by other means, no agreement between duplicates can be expected.

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ELECTROLYTIC DECALCIFICATION BY MEANS OF A ONE-DAY BATTERY CHARGER

P. T. DOLAN, M.D.
AURORA, ILL.

WE HAVE found the metallic rectifier type of battery charger completely satisfactory for bone decalcification, and have found it to be considerably less expensive than commercial apparatuses now on the market. The apparatus is easily set up with materials readily available in any laboratory. The battery charger* used delivers an amperage ranging up to approximately 2.0 using alternating current. An 8 ohm variable resistor may be used for control if desired.

In conjunction with this charger we use a 4 by 4 inch glass icebox dish fitted with plastic lid. Platinum wire electrodes are fitted in the lid in such a manner that they are four inches apart and extend to within one-half inch of the bottom of the dish. The electrodes are fitted to the plastic lid by means of small bolts to which the positive and negative poles of the charger can be attached. Twenty gauge platinum wire is used. The bone sections are attached to the positive pole wire by simple wrapping in the case of intact fragments. Smaller fragments are placed in a small basket devised from ordinary plastic window screen through which the platinum wire is run.

The solution used is that of Richman and associates, as suggested by Ducey and Shippy,[1] consisting of concentrated hydrochloric acid, eight parts; formic acid, chemically pure, ten parts; distilled water, eighty-two parts. We have found 300 cubic centimeters to be a sufficient solution for one specimen. The maximum current obtained is about two amperes, which in our experience did not raise the temperature beyond 30° C.—a safe temperature from the standpoint of tissue coagulation.

Specimens of cortical bone approximately 0.3 cm. in thickness are decalcified within twenty-four hours; specimens consisting of only spongy bone of similar thickness are decalcified as a rule within three to six hours; tiny fragments must be checked within two to four hours.

COMMENT

This apparatus, besides being eminently satisfactory for the purpose intended, has the added advantages of cheapness, and ease in construction, and is without the hazard of high temperatures which might coagulate the tissues. The staining of tissues decalcified in this manner is in every way comparable to the staining obtained after using ordinarily accepted techniques.

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